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13. ABSTRACT (Maximum 200 words)

This project was a follow-on to the original phase of development of freeze-dried platelets for transfusion under prior ONR grant N00014-89-J-1712. During the 3 1/2 year performance period, we met our goals of evaluation of a standard method of stabilizing and lyophilizing blood platelets from human or animal sources in several in vitro and in vivo test systems. The mechanism of platelet adhesion was found to be intact in our lyophilized platelet preparations, and evidence of residual metabolic capability was demonstrated. Two different long-term storage studies (up to 1 year) were carried out at various temperatures, which showed some instability at 22°C but none at 4°C or -70°C. Scale-up of production with sterile technique was begun in our research labs but transferred to Armour Pharmaceutical Corp. as they entered the ATD program. The in vivo hemostatic efficacy of rehydrated platelet preparations was demonstrated in several animal models involving normalization of a prolonged bleeding time in thrombocytopenic rats or rabbits, and in von Willebrand's disease or normal dogs. Labelled rehydrated platelets were found histologically in wound sites in infused dogs. These findings show the potential utility of these preparations in transfusion medicine.

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Dear Sir,

Please find enclosed two copies of the Final Technical Report for the period February 1, 1992 - June 30, 1995. If you have any questions I may be contacted at 919-816-5020. Thank you.

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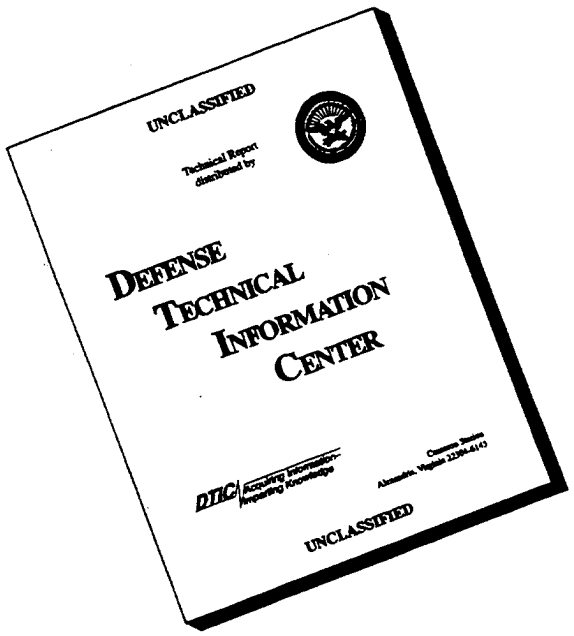

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Final Technical Report:

Grant# N00014-92-J-1244

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Performance Period: February 1, 1992 - June 30, 1995 (with no-cost extension)

"Evaluation of Dried Storage of Platelets for Transfusion with emphasis on Physiologic Integrity and Hemostatic Functionality"

submitted Jan. 24, 1996 by

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A. Performance of Project

1. Introduction

This project was an extension and expansion of the work done under grant # N00014-89-J-1712 to develop a preparation technique for freeze-drying blood platelets for use as a transfusion medicine resource in combat casualty care. The goals for this period centered on testing the best preparations developed under the initial grant in a variety of in vitro and in vivo hemostatic challenges. The work was divided over two performance sites: East Carolina University in Greenville, N. C. and The University of North Carolina at Chapel Hill. Much of the in vivo work was carried out at Chapel Hill in the canine, swine and rat colonies while the investigations at ECU focussed on in vitro evaluations of platelet structure and function. During the final year of performance, a collaborative study was carried out at McMaster University in Hamilton, Ontario, with infusion of our human lyophilized platelet preparations into a well-established hemostasis model in thrombocytopenic, immunocompromised rabbits.

The investigators involved during this project were:

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Morris A. Blajchman, MD, Ph.D., (collaborator) Professor, Dept Pathology,
McMaster University, Hamilton, Ontario

In addition to the above academic faculty, industrial scientists from Armour Pharmaceutical Corp. (Kankakee, IL) became involved with the overall project (but not in the direct performance of our specific aims) to pursue pharmaceutical production of lyophilized platelets with our technology. The parent company of Armour, Rhone-Poulenc Rorer signed a licensing agreement with ECU and UNC under the patent application filed in the course of grant N00014-89-J-1712 to protect the intellectual property developed in this project. Fred Feldman, Ph.D., Vice-President of research and development directed the team for Armour/RPR.

2. Goals

The specific aims of the proposal are listed below. Although these aims were ostensibly completed, several important new issues and questions have arisen out of this work. Further preclinical investigation and clinical totals will be necessary to determine the ultimate value of our approach to preparing freeze-dried platelets for use in military or civilian blood banking.

(1) To test the hemostatic potential of labelled, rehydrated autologous platelets in animal model systems of thrombosis and hemostasis.

Freeze-dried homologous animal platelet preparations were infused into Sprague-Dawley rats made thrombocytopenic by an anti-platelet antiserum. Assessment of the hemostatic effect of the infusion included platelet counts and a bleeding time test in the toenail. It was also found that human platelet preparations could be tested similarly in the rat, but had shorter life spans in circulation than homologous cells; performance of lyophilized human platelet preparations in this model of hemostasis were thus compared to that of fresh human platelets. Infusions of lyophilized canine platelets were carried out in normal dogs and several animals in a colony bred for the von Willebrands disease trait. Effect of the infusions was assessed by reduction in the ear bleeding time and cessation of spontaneous hemorrhage. In several experiments, the rehydrated lyophilized canine platelets were labelled with the fluorescent dye PKH 26 (Zynaxis Corp.) and tracked in histological sections of tissue from ear bleeding time wound sites in the infused dogs to demonstrate involvement of the rehydrated platelets in hemostatic plug formation. In a third in vivo, test of hemostasis, lyophilized human platelet preparations of several different protocols (variations of the stabilization step as 1.8% paraformaldehyde for 45, 60, or 120 minutes, or 0.02% permanganate for 10 minutes) were sent to Dr. Morris Blajchman at McMaster University in Hamilton, Ontario, for infusion in his well-established model of thrombocytopenic rabbits which are immunocompromised with ethyl palmitate so that xenographic cells are not immediately removed from circulation. Hemostatic effectiveness of the infusates was assessed by platelet count increments and by ear

bleeding time tests performed at 1 hour post-infusion. Performance of the lyophilized platelets was compared to controls of infusions of fresh human platelets. Pilot studies were also performed in other test systems, including a canine model of platelet involvement in thrombus formation after arterial stenosis, and attempts were made to track the circulatory lifespan of fluorescent labelled canine rehydrated platelets (PKH 26 or other dyes) by flow cytometry in samples taken and stabilized at UNC-CH then shipped to ECU for analysis.

(2) To examine the mechanisms of adhesion of rehydrated lyophilized platelets to thrombogenic denuded blood vessel strips in the Baumgartner perfusion chamber.

Both UNC-CH and ECU set up annular perfusion chamber systems, and used complementary analytical techniques to evaluate the adhesion of rehydrated lyophilized platelet preparations to canine or porcine vessel strips deliberately denuded of endothelium by manipulation and exposure to air. Perfusion of the vessel with whole blood at high flow rate (100-130 mL/min) forced the adhesion of platelets to the vessel to be shear-resistant and dependent on von Willebrands factor. Coverage of denuded sites on the vessel subendothelium surface was assessed by scanning electron microscopy (UNC-CH) or immunofluorescence microscopy (ECU). Running several chambers in the same day gave an experimental design for comparison of results among fresh whole blood, rehydrated platelets, and stored blood bank platelets. Activation-related changes were noted in the adherent (and non-adherent) platelets by morphology, flow cytometry of neo-antigens, procoagulant activity assay, and RIA of Thromboxanes produced during perfusion. Attempts to modulate adhesion of platelets to the vessel included addition of PGE-1 or aprotinin, or addition of aspirin or indomethacin to block Thromboxane production. Several runs were also performed in the Baumgartner system with nearly intact vessel strips (not deliberately denuded) to test for inappropriate adhesion of rehydrated platelets to non-thrombogenic sites.

(3) To study the interaction of rehydrated platelets with activators and regulators of the fibrinolytic system.

Preliminary experiments were performed with flow cytometry to identify F. XIII expression on the surface of rehydrated platelets by immunofluorescence under a variety of experimental conditions. It proved difficult to standardize a fibrinolysis test system in which to measure the activity or effect of F. XIIIa or PAI released from rehydrated platelets. Instead, this goal was advanced in a new ATD project (N00014-93-I-1034) as an element of evaluation of the functionality of infused rehydrated platelets in a canine model system of hemorrhage after extended cardiopulmonary bypass circulation +/-AMICAR (plasmin inhibitor).

(4) Metabolism of rehydrated platelets-active or inert?

We gained information from the Baumgartner perfusion experiments in Aim (2) that the rehydrated platelets were capable of expressing certain aspects of in situ

activation, including TxA_2 production. Experiments were also carried out to show by electron or light microscopy that the platelets were capable of spreading and undergoing a morphological change of shape on formvar coated grids, glass slides, or on the denuded vessel strips of the Baumgartner chamber. Earlier efforts showed a residual response of rehydrated platelets in the metabolically-driven test of extruding water after hypotonic shock, but controls showed that this level of response could also be obtained with isotonic solutions. Other experimental constructs showed that the rehydrated platelets could be stimulated by thrombin/collagen to become more procoagulant, and could become involved in aggregates of fresh platelets in response to strong agonists. Only indirect evidence of responsiveness of the rehydrated platelets could be obtained. Resources not utilized in Aims 3 and 4 were re-allocated to support a new approach to in vivo testing of hemostasis, namely Dr. Blajchman's thrombocytopenic rabbit model, which had not been listed in the original set of aims.

(5) Scale-up of dried platelet production.

We were able to process as much as six units of citrated blood (three liters) from pooled canine or porcine collections at UNC or ECU to produce large batches of lyophilized platelets. Several attempts were made to process whole units of human blood in a "closed system" such as in the triple-bag connected collection set, or in open containers under a sterile laminar-flow hood to approach the practical parameters faced in GMP scale-up to a pharmaceutical product. However, when interest and activity of Armour Pharmaceutical Corp. in this project was initiated, UNC-CH and ECU gave over the scale-up efforts to the Armour development group. Their scientists came on several occasions to our laboratories to learn the developed technology on handling platelets on our smaller scales and then applied their efforts to GMP and GLP procedures of handling large volumes of blood. Their development is still on-going.

(6) Testing of storage conditions.

As the platelet processing steps became more established (% paraformaldehyde or permanganate, choice of buffers, bulking materials, etc.), vials of dried platelets were set aside for analysis in the latter phases of this projects. Two separate deliberate 12 month studies also were set up with different types of preparations and stored in desiccators at room temperature or 4^0C, or stored frozen at -70^0C. Comparison tests included morphology, procoagulant activity, responsiveness, and hemostasis testing under low volume conditions (like in the new Clot Signature Analyzer for in vitro thrombus formation under flowing conditions). A more thorough evaluation of the storage stability of the lyophilized platelet preparations will require better control of the residual water content since this parameter appears to be very significant but cannot be easily controlled in small-scale preps.

(7) Alternative strategies for stabilization.

Further work on alternatives to using paraformaldehyde to stabilize the platelets

pre-lyophilization included use of permanganate or changes in bulking reagents, such as sucrose or trehalose for albumin. None of these alternatives protocols has succeeded in preparations that out perform or even equal the now-standard PARA21 preps that Armour is developing from our techniques. Preliminary work performed under other ATD projects with UNC, ECU, and Armour has shown that the paraformaldehyde processing may serve not only as a stabilizing step but also as a new means to sterilize the product due to its bactericidal and viricidal properties as used in our preparations. This highly desirable property made pursuit of alternative stabilization protocols less attractive.

B. Results

Data and interpretation from experiments performed under the goals of this project are given in the attached manuscripts, abstracts, and subcontract final report. The summary of findings in brief is that we have refined the preparation technique for small-scale preparation of fixed lyophilized platelets which retain the following properties and characteristics upon rehydration:

(a) morphology - mostly discs and spheres, with some pseudopodia; approximately equivalent to a fresh platelet concentrate from the blood bank

(b) procoagulant activity - expresses prothrombinase activity in vitro in PRP or PF3 clotting assays, inducible further with strong agonists

(c) metabolic activity - not totally inert; appear to produce TxA_2, change shape (spreading on surfaces), and express activation neo-antigens to a degree after stimulation but less than that of fresh platelets

(d) adhesion - nearly equivalent to fresh platelets in Baumgartner perfusion chamber, not inappropriately thrombotic on intact endothelium

(e) activation response - although not capable of macroscopic aggregation, see (b) and (c) above

(f) hemostasis - shown to shorten the bleeding time and concentrate at injury sites in three different animal models of hemorrhage or thrombocytopenia

(g) circulatory lifespan - not yet known; hard to label

(h) pharmaceutical suitability - acceptable to Armour/Rhone-Poulenc Rorer

(i) storage stability - probably >1 year at -70°C or 4°C

Although more investigation needs to be done and is underway under grant N00014-93-I-1034 and other efforts, we believe that the progress to-date shows the feasibility of producing freeze-dried platelet preparations that will be of value in augmenting transfusion resources for combat casualty care or civilian transfusion medicine.

C. Publications, Presentations, Patents

The following lists show the papers, abstracts, and presentations of data generated under this project. Abstracts outnumbered papers greatly because of the need to avoid "enabling" publication of preparation details while processing the patent application and agreements with Armour Pharmaceutical. Several more journal article submissions will be made on these data in the near future now that filings have been completed.

1. Journal articles:

   "Preservation of hemostatic function and structural properties of rehydrated lyophilized platelets: Potential for long-term storage of dried platelets for transfusion"; MS Read, RL Reddick, AP Bode, DA Bellinger, TC Nichols, KK Taylor, SV Smith, DK McMahon, TR Griggs, KM Brinkhous
   Proc. Natl. Acad. Sci. 92: 397-401, 1995

   "Platelet Storage: Efforts to Extend the Shelf Life of Platelet Concentrates", MS Read, AP Bode
   Molecular Medicine Today 1 (7):322-328, 1995.

   One other manuscript in review/revisions
   Two other manuscripts in preparation

2. Scientific meeting abstracts

   "Adherence and activation of rehydrated platelets in Baumgartner perfusion chamber"; AP Bode, MS Read, RL Reddick
   Transfusion 33(9S, #S280): 72s, 1993

"Transfused rehydrated platelets support hemostasis and thrombosis"; MS Read, RL Reddick, TC Nichols, DA Bellinger, AP Bode, KK Taylor, KM Brinkhous, TR Griggs
Blood 82(Suppl. 1, #623):159a, 1993

"Hemostatic properties of lyophilized platelets in tests of bleeding times"; AP Bode, MS Read, RM Lust
Transfusion 34(10S, #S307): 74s, 1994

"Hemostatic properties of human lyophilized platelets in a thrombocytopenic rabbit model and a simulated bleeding time device"; AP Bode, MA Blajchman, L Bardossy, MS Read
Blood 84(Suppl.1, #1840): 464a, 1994

"Promotion of platelet adhesion to thrombogenic sites by microparticles in stored platelet concentrates"; LY Yang, AP Bode
Blood 84(Suppl.1, #1885): 475a, 1994

"A stable radiolabel for fresh and dried platelets"; RJ Kowalsky, KK Taylor, DK McMahon, ME Brecher, DA Bellinger, RL Reddick, MS Read
Blood 84(Suppl. 1, #1278): 323a, 1994

"Long-term stability of lyophilized platelets: physical integrity and hemostatic function"; AP Bode, MS Read
accepted for publication in Transfusion for Nov. '95

3. Presentations of data

Canadian Red Cross annual meeting, @Hamilton, Ontario; May '93
Site visit ONR/Armour/UNC-CH/ECU, @Chapel Hill, NC; Aug "93
American Association of Blood Banks, @Miami Beach, FL; Oct '93: 1 oral
American Society of Hematology, @St. Louis, MO; Dec. '93: 1 poster
ONR Project Review, @the Naval Research Lab, Arlington, VA; May '94
British Blood Transfusion Society, @Southampton, UK; Sept. '94
American Association of Blood Banks, @San Diego, CA; Nov. '94: 1 oral
American Society of Hematology, @Nashville, TN; Dec. '94: 3 posters
ONR Project Review, @the Naval Research Lab, Arlington, VA; April '95
NIH Workshop: Microbes in Blood Products, @Washington, DC; Sept. '95
American Assoc. of Blood Banks, @New Orleans, LA; Nov. '95:1 oral

4. Patent activity

The original patent application for commercial production of our lyophilized platelet preparations for transfusion medicine was prepared during the course of a previous ONR grant (N00014-89-J-1712) and filed with the US Patent Office on May 29, 1992 (serial# 07/891,277). Since that filing date, more data and claims have been appended (July '92, April '93) and a filing was made under the CIP agreement in April '93. The initial action of the patent examiner was to reject all claims, mostly on the basis of prior obvious art and enablement from disclosures they considered public in the technical reports distributed under this grant to the federal government. This interpretation is being challenged by the patent attorneys. In Nov. '92, an option for license agreement was signed with Armour Pharmaceutical (Rhone-Poulenc Rorer) and was exercised in May '94 to provide Armour with an exclusive license to produce the technology disclosed in the patent application. At that point, Armour assumed the obligations to pursue patent rights and further filings. Patent applications have since been filed in Mexico (May '93) and Australia, Canada, Europe, and Japan (Oct. '94). Further action is in preparation to rebut the original rejection of claims by the US Patent Office.

Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 397–401, January 1995
Medical Sciences

Preservation of hemostatic and structural properties of rehydrated lyophilized platelets: Potential for long-term storage of dried platelets for transfusion

(platelet adhesion/platelet agglutination/thrombocytopenia/thrombosis)

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Contributed by Kenneth M. Brinkhous, September 22, 1994

ABSTRACT **Currently, therapeutic platelet concentrates can be stored for only 5 days. We have developed a procedure that permits long-term storage of fixed and lyophilized platelets that retain hemostatic properties after rehydration. These rehydrated lyophilized platelets (RL platelets) restore hemostasis in thrombocytopenic rats and become incorporated in the hemostatic plug of bleeding time wounds of normal dogs as well as von Willebrand disease dogs with partially replenished plasma von Willebrand factor. Ultrastructurally, these platelets are well preserved and are comparable to control normal washed platelets. Flow cytometry analysis shows that RL platelets react with antibodies to the major surface receptors, glycoprotein (GP)Ib and GPIIb/IIIa. These receptors are involved in platelet agglutination, aggregation, and adhesion. *In vitro* functional tests document the ability of RL platelets to adhere to denuded subendothelium and to spread on a foreign surface. Circulating RL platelets participated in carotid arterial thrombus formation induced in normal canine subjects. The participation of RL platelets in these vital hemostatic properties suggests that with further development they could become a stable platelet product for transfusion.**

To promote effective hemostasis, platelets must respond quickly to changes in normal blood flow or vessel injury (1, 2). After vascular injury, platelets adhere to exposed subendothelium, aggregate, and form a primary platelet plug. Platelet activation and initiation of coagulation follow with stabilization of the platelet plug by the formation of fibrin. The initiation of a thrombus at a site of vascular injury is mediated through platelet membrane glycoprotein (GP) receptors (3, 4). Platelet adhesion to a damaged vessel wall and its extracellular matrix at high shear is primarily mediated through the specific interaction of the platelet membrane GPIb–IX complex and bound von Willebrand factor (vWF) (5–7), which is synthesized and released into plasma and the vessel wall by endothelial cells (1). Platelet adhesion at low shear rates is mediated by several interactions, including collagen with the $\alpha_2\beta_1$ integrin (7). Platelet adhesion stimulates a spreading of the platelet (8). Although the mechanism of platelet spreading has not been completely characterized, recent *in vitro* studies have shown that platelets will spread on surfaces coated with fibrinogen (9) or polymerized fibrin (10). The activation of the GPIIb/IIIa receptor by agents such as ADP results in a conformational change in the receptor (11–13). The activated receptor binds fibrinogen, which forms a "bridge" between the platelets, and causes aggregation (1, 14, 15). Activated platelets provide the phospholipid surface for the assembly of blood clotting enzyme complexes, and the concentration and localization of activated coagulant proteins at sites of vessel wall injury may be facilitated by adherent platelets (16). Internal storage granules in platelets release clot-promoting contents in response to activation of biochemical systems triggered by platelet–platelet or other interactions. Interactions of adherent platelets with neutrophils, mediated through platelet integrins, specifically P-selectin receptor (17), may contribute to hemostatic and other cell functions (13).

The control of hemorrhage due to thrombocytopenia often requires transfusion of multiple units of fresh platelets. In transfusion medicine, platelets cannot be replaced by other blood products or artificial media. Maintenance of critical membrane GPs during storage is crucial to platelet function *in vivo*. With the storage life of fresh platelets limited to 5 days, there has been considerable study to lengthen platelet shelf life and enhance stored platelet response (18–24). Investigators have addressed storage conditions that preserve platelet integrity and responsiveness (25, 26). The effects of preservatives on platelet activation and expression of membrane GPs have also been investigated (27). In a recent review, the use of inhibitors of platelet activation to extend the shelf life and enhance the quality of liquid stored platelets is discussed (28). Cryopreservation of platelets extends the shelf life to 1 year but requires extensive washing and processing to remove cryoprotectant agents (29). In other blood cell studies, red blood cells washed with saline followed by lyophilization retain metabolic activities similar to red blood cells stored under blood bank conditions (30). Lyophilization of platelets or platelet-rich plasma as previously attempted (31–33) neither preserved the structural integrity of the platelets nor provided adequate hemostasis when infused into thrombocytopenic pediatric patients or hemorrhagic animal models.

We have successfully prepared a paraformaldehyde-treated, lyophilized and rehydrated platelet product (RL platelets) with intact morphology and agglutinating properties (34–36). Efforts to refine this process have produced platelet preparations that are structurally stable and capable of undergoing activation. We report here on the hemostatic properties displayed by our RL platelets as tested *in vitro* and in canine and rat animal models. In this study, we have administered RL platelets to normal and von Willebrand disease (vWD) dogs and to thrombocytopenic rats. Our results indicate that RL platelets retain many essential biologic properties and promote hemostasis. The successful preparation of a dried transfusion platelet product without loss of hemostatic capabilities

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Abbreviations: RL platelet, rehydrated lyophilized platelet; BT, bleeding time; vWD, von Willebrand disease; vWF, von Willebrand factor; GP, glycoprotein.

‡To whom reprint requests should be addressed.

suggests potential for the development of this product as a blood banking resource.

MATERIALS AND METHODS

Preparation of RL Platelets. Lyophilized human and canine platelets were prepared as described (34). Human platelets were obtained from the American Red Cross 3–6 days after collection. Canine platelets were obtained from normal dogs (n = 5) from the Francis Owen Blood Research Laboratory (University of North Carolina). Washed platelets were incubated for 1 hr with paraformaldehyde at concentrations of 1.8% for human platelets and 0.68% for canine platelets. Washed paraformaldehyde-free platelets in citrated saline (0.006 M trisodium citrate/0.154 M NaCl, pH 6.8) with 5% bovine serum albumin were frozen in 1-ml aliquots containing 8×10^8 platelets per ml and lyophilized at −20°C to −40°C for 20–24 hr. Dried platelets were stored at −80°C until used. Dried platelets were rehydrated in 1.0 ml of imidazole buffer (IB; 0.084 M imidazole, pH 7.35) and centrifuged at 1000 × g for 8 min to pellet the platelets. The rehydrated platelets were freed of albumin and imidazole by three washes in citrated saline. For use, the platelet pellets were resuspended in platelet-poor plasma or in a modified Hanks' buffered salt solution (mHBSS; 0.17 M NaCl/6.7 mM KCl/1.0 mM $MgSO_4$/0.5 mM K_2HPO_4/2.8 mM Na_2HPO_4/13.8 mM dextrose, pH to 7.2 with 1.4% $NaHCO_3$) for *in vitro* studies and in normal saline for *in vivo* studies. Gas chromatography was used to document the absence of formaldehyde in washed canine and human RL platelet solutions. A detection limit of 0.002% was used (National Medical Laboratories, Willow Grove, PA).

RL platelets were labeled with the fluorescent dye Zynaxis PKH 26 as a marker for platelets in infusion studies. The pelleted rehydrated platelets were washed once by resuspension in 1.0 ml of acid citrate dextrose, centrifuged at 600 × g for 8 min, and resuspended in 0.1 ml of mHBSS. The mixture was incubated for 20 min in the dark and centrifuged (600 × g; 8 min). The labeled platelets were washed once by resuspending in 1.0 ml of mHBSS containing either 0.1% canine serum albumin or 0.1% bovine serum albumin. No label was transferred or lost from fluorescent platelets incubated at 25°C–37°C for several hours in whole blood.

Hemostatic and Other Methods. The saline bleeding time (BT) in canines was performed as described (37). The BT wound sites were excised and prepared for fluorescence and light microscopy (38). For the rat toenail BT, rats were anesthetized with ketamine hydrochloride (Ketaset)/ProMACE, and a foot was antiseptically cleansed and warmed in a 37°C bath. A sterile scalpel blade was used to excise the distal 1.0 mm of the vascular nail bed from one nail. Blood was blotted onto filter paper for BT measurements. Platelet adhesion of fresh and RL human platelets was compared in an annular perfusion chamber (39). Adhesion studies were carried out at high shear (flow rate, 125 ml/min; 37°C) using porcine arterial subendothelium that had been denuded by exposure to air. After platelet solutions containing fresh or RL platelets were exposed to the subendothelium, segments were removed and processed for scanning electron microscopy to visualize platelet adhesion. Citrated blood, platelet-rich plasma, platelet-free plasma (PFP), and red blood cell fractions were isolated as described (34). Adhesion of fresh platelets was determined after passing whole citrated blood over the subendothelium. Adhesion of RL platelets was determined after PFP enriched with RL platelets and the red blood cell fraction was passed over the subendothelium. To confirm the absence of platelets in PFP prior to the addition of RL platelets, phase-contrast microscopy was used. To examine platelet spread, RL platelets were reconstituted in IB, washed once in HBSS (40) to remove albumin, and spread on Formvar-coated grids (41). Spread platelets were examined with a Cambridge autoscan scanning electron microscope at 20 kV.

For morphological studies, fresh and RL platelet pellets were processed for transmission electron microscopy as described (38). Platelets were examined with a Zeiss 10A microscope. Rehydrated platelet surface antigen distribution and overall light scatter properties were analyzed on a Becton Dickinson FACS 440 flow cytometer. Monoclonal antibody binding to rehydrated platelets or to fresh platelets resuspended in citrated plasma was evaluated by indirect immunofluorescence 488-nm excitation as described (42). Control antibody was used to identify nonspecific IgG binding to fresh and rehydrated platelets. In each run, 10,000 events were measured and analyzed.

Infusions of RL Platelets. The animals used were normal dogs (n = 3), a vWD dog (n = 1), and Sprague–Dawley rats (n = 3). The vWD dog was from the closed colony at the Francis Owen Blood Research Laboratory. Normal Sprague–Dawley rats were obtained from the Division of Laboratory Animal Medicine (University of North Carolina, Chapel Hill). All animals were treated according to published standards (43). Thrombocytopenic rats were given 4.5×10^9 and 3.4×10^{10} human RL platelets, respectively, through the tail vein. Toenail BTs were performed immediately after infusion of RL platelets. Normalization of BT was taken as an indicator of RL platelet hemostatic function.

Fluorescence-labeled canine RL platelets were infused into three normal dogs. After infusion of these platelets, plasma levels of vWF (44), coagulation factor IX (45), coagulation factor VIII (46), platelet counts (Unopette; Becton Dickinson), and serum fibrin degradation products (Thrombo-Wellcotest; Wellcome) were determined prior to and at the

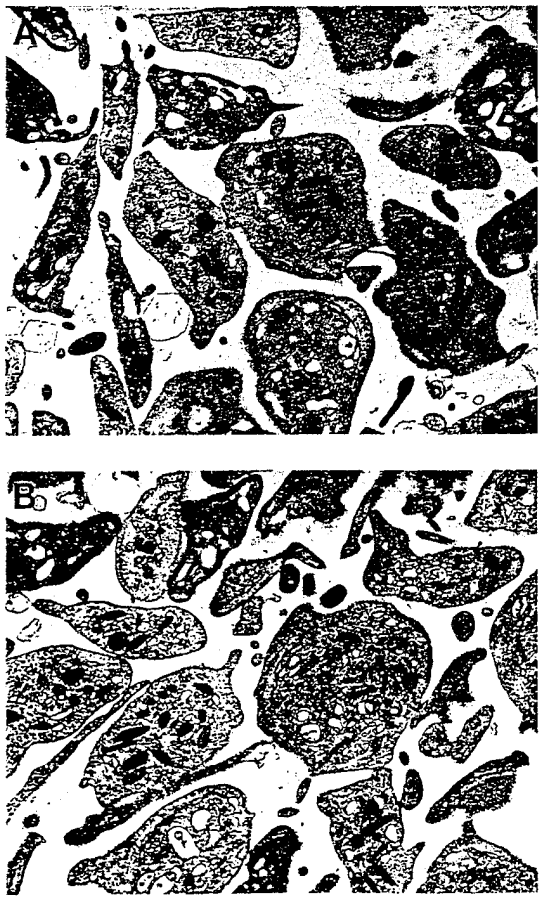

FIG. 1. Transmission electron microscopy of fresh (*A*) and RL (*B*) platelets. Both fresh and RL platelets have intact and randomly distributed organelles and some pseudopod formation. (×7760.)

Table 1. GPIb, GPIIb/IIIa, and GPIb/IX on the surface of fresh and RL platelets

Antigen	Antibody	% labeled platelets	
		Fresh	RL
GPIb	AN-51	98	92
GPIb	SZ-2	92	85
GPIb/IX	SZ-1	98	92
GPIIb/IIIa	10ES	98	98

Monoclonal antibodies to GPIb (clones AN-51 and SZ-2) were obtained from Dakopatts (Glostrup, Denmark) and AMAC (Westbrook, ME), respectively. The anti-GPIb/IX complex antibody (SZ-1) and the anti-GPIIb/IIIa complex antibody (clone 10E5) were obtained from AMAC and Barry Coller (State University of New York, Stony Brook), respectively. There were <5% labeled platelets in the positive gates using a control nonimmune mouse IgG-2a antibody (Coulter Immunology). Fresh platelets were washed with citrated saline.

following intervals postinfusion: 1, 5, 15, 30, and 60 min during the first hour, and 2, 4, 6, 8, and 24 hr thereafter. BTs were performed postinfusion of labeled RL platelets and wounds were excised after cessation of bleeding for examination by fluorescence and light microscopy (38). A similar infusion was performed with a vWD dog after partial replacement of vWF by treatment with cryoprecipitate. RL platelets represented 51.4% of the vWD dog's normal platelet count based on the number of RL platelets infused. Cryoprecipitate was prepared as described (47) and assayed for vWF content (44). A modified Folts procedure (48) was used to produce carotid arterial thrombosis in dogs (n = 3).

RESULTS AND DISCUSSION

Structural Features of RL Platelets. Transmission electron microscopy showed that RL platelets are morphologically similar to fresh washed platelets (Fig. 1). RL platelets are partially activated, similar to fresh washed platelets. Flow cytometry using anti-GPIb and anti-GPIIb/IIIa monoclonal antibodies indicated that both GPs were present on the surface of RL platelets (Table 1). The number of RL platelets with antibody recognition of receptors is expressed as a percentage of the platelets with specific immunofluorescence.

Functional Characteristics of RL Platelets *in Vitro*. Platelet adhesion and platelet spreading are shown in Fig. 2. A comparison of platelet adhesion with fresh and RL platelets shows that RL platelets adhere in numbers similar to fresh platelets, with irregular shapes and with multiple pseudopodia,

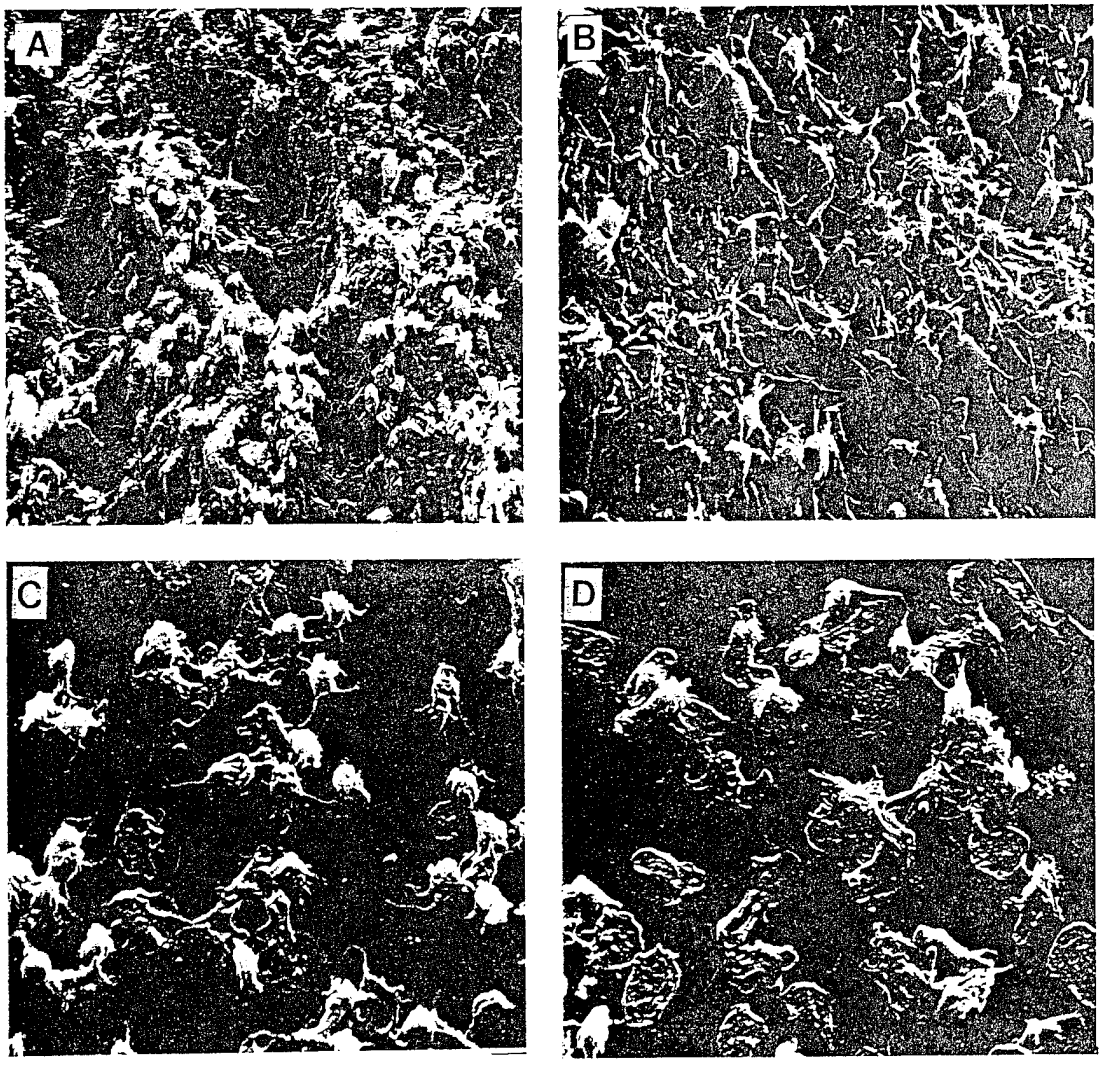

FIG. 2. Scanning electron microscopy (SEM) of vessel segments from an annular perfusion chamber with RL platelets (*A*) and fresh platelets (*B*). Vessel subendothelium exposed to platelet-free blood was free of platelets (data not shown), while segments exposed to platelet-containing blood was carpeted with platelets. SEM of spread RL platelets shows that paraformaldehyde-stabilized platelets adherent to Formvar-coated grids formed dendritic patterns with multiple pseudopodia and are fully spread (*C*), similar to fresh platelets spread on the same substrate (*D*). (*A*, ×960; *B*, ×2320; *C*, ×2080; *D*, ×1600.)

Table 2. Infusions of human RL platelets shorten the prolonged BTs in thrombocytopenic rats

	Normal		Thrombocytopenic		Thrombocytopenic with RL platelets	
Animal	BT, min	Platelet count per μl, $\times 10^{-3}$	BT, min	Platelet count per μl, $\times 10^{-3}$	BT, min	Platelet count per μl, $\times 10^{-3}$
1	0.5	685	>15	25	0.5	220
2	2.0	580	>15	32.5	1.5	237

Thrombocytopenia was induced in two Sprague–Dawley rats by treatment with 1 ml of a 1:10 dilution of anti-rat thrombocyte polyclonal antibody (Accurate Chemicals). Platelet counts and toenail BT measurements were used to monitor the level of circulating rat platelets 10 min after treatment with the antibody. RL platelets were infused immediately after a BT of >15 min was established. In a control rat without treatment with RL platelets, bleeding times were >15 min, and platelet counts were <50,000 platelets per μl for >12 hr.

although pseudopodia are present to a lesser extent in RL platelets (Fig. 2 *A* and *B*). Neither RL platelets nor fresh platelets were present in areas where the endothelium remained intact (data not shown). A comparison of platelet spreading of fresh and RL platelets showed both having a similar flattened or "pancake" morphology (Fig. 2 *C* and *D*). Multiple pseudopodia were found associated with both fresh and RL platelets, which were not completely spread. Few discoid forms were present, suggesting that paraformaldehyde-stabilized platelets retained sufficient metabolic activity for platelet spreading to occur. Earlier studies have demonstrated that functional GPIb is preserved in lyophilized platelets (49). While GPIIb/IIa epitopes are identified in RL platelets, minimal platelet aggregation was observed in preliminary studies with ADP (A.P.B., unpublished data).

Functional Characteristics of RL Platelets *in Vivo*. RL platelets were labeled with a fluorescent dye to distinguish rehydrated platelets from circulating native platelets in infusion experiments. BT studies in rats with human RL platelets and in normal and vWD dogs with canine RL platelets were conducted. The results of two separate RL platelet infusion experiments using thrombocytopenic rats are shown in Table 2. After administration of human RL platelets, toenail BTs in two rats treated with an anti-rat thrombocyte antibody decreased from >15 min to normal. One rat was tested at 30 min and had a toenail BT of 3.5 min, which remained corrected for 1 hr, at which time the rat was sacrificed. Treatment of normal rats with diluted anti-rat thrombocyte antibody depleted circulating rat platelets to <33,000 platelets per μl and lengthened the rat toenail BT to >15 min. Normal rats treated with experimental levels of the anti-rat thrombocyte antibody without additional RL platelets had elongated BTs (>15 min) and low autologous platelet counts (<50,000 platelets per μl) for 12 hr. The toenail BT is a simple and reproducible method of measuring BT in the rat and requires only that the rat be anesthetized before testing. In 10–12 normal rats, the BTs ranged from 30 sec to 3 min.

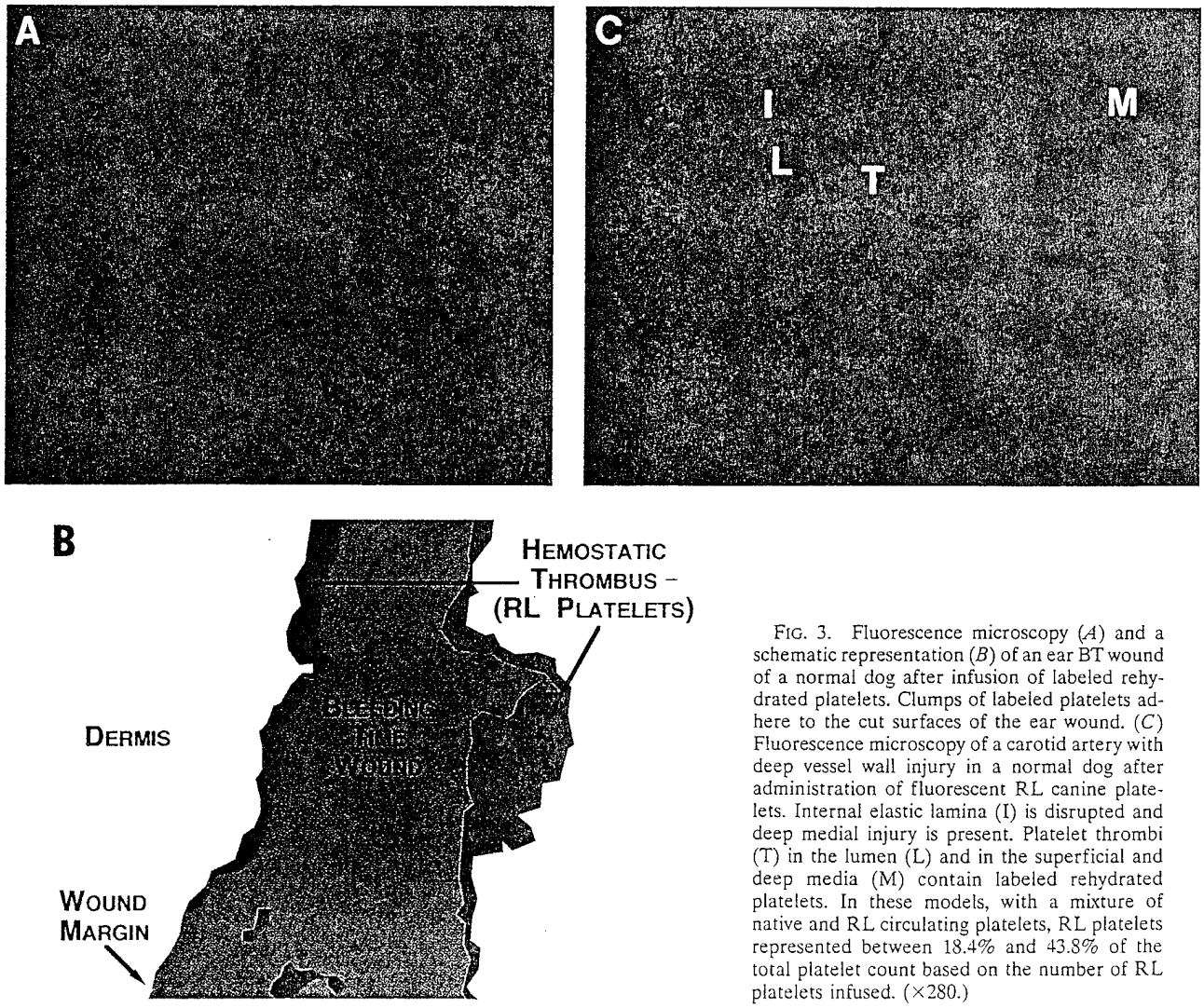

FIG. 3. Fluorescence microscopy (*A*) and a schematic representation (*B*) of an ear BT wound of a normal dog after infusion of labeled rehydrated platelets. Clumps of labeled platelets adhere to the cut surfaces of the ear wound. (*C*) Fluorescence microscopy of a carotid artery with deep vessel wall injury in a normal dog after administration of fluorescent RL canine platelets. Internal elastic lamina (I) is disrupted and deep medial injury is present. Platelet thrombi (T) in the lumen (L) and in the superficial and deep media (M) contain labeled rehydrated platelets. In these models, with a mixture of native and RL circulating platelets, RL platelets represented between 18.4% and 43.8% of the total platelet count based on the number of RL platelets infused. (×280.)

RL platelets were infused into three normal dogs and one vWD dog in order to determine whether the RL platelets were incorporated into the hemostatic thrombi of BT wounds. The vWD dog was infused with cryoprecipitate, which raised the vWF level to 38% of normal; the BT of the vWD dog was reduced from >15 min to 8 min. RL platelets were then administered to the vWD dog, and there was no significant change in the BT. The BT remained in the normal range (6 min) for the other three dogs. RL platelets circulated for the duration of the experiments (up to 4 hr). They were found to be part of the hemostatic plug in normal dogs (Fig. 3*A* and *B*). In the vWD dog, RL platelets were observed at the same sites as in the normal dogs but in far fewer numbers (data not shown). Samples of liver, lung, and kidney were examined after sacrifice of these animals. No gross or microscopic changes were observed.

Carotid arterial thrombosis was induced by using a canine model of stenosis and injury in three normal dogs that had been infused with RL platelets (48, 50). All animals experienced occlusive thrombosis as indicated by the cessation of blood flow. When thrombosis occurred, the vessels containing the thrombi were harvested after >30 min of observation and examined by fluorescence microscopy. Fluorescent RL platelets were present in the induced thrombi and were also adherent to the exposed subendothelium. Single and aggregated platelets were present in areas of hemorrhage (Fig. 3*C*). In vessels where damage was minimal, fluorescent RL platelets were seen in the lumen and adhering to the luminal surface where the internal elastic lamina was disrupted. There was no evidence of RL platelets adhering to intact endothelium. The absence of disseminated intravascular coagulation was indicated by no change in fibrinogen level, no loss of coagulation factors VIII and IX, and no appearance of fibrin degradation products.

Platelet preparations can be stored for several days without the use of refrigeration. After this period, platelets lose many of their functions (51, 52). Lyophilization has the potential to extend blood cell (erythrocytes and platelets) shelf life from days to years. The fixation of platelets in paraformaldehyde followed by lyophilization has proven effective in maintaining some of the normal functions of the human platelet (34). We have shown that RL platelets retain many properties necessary for normal hemostasis. These observations suggest that this method of stabilization may offer a method for long-term storage of platelets.

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ADHERENCE AND ACTIVATION OF REHYDRATED PLATELETS IN BAUMGARTNER PERFUSION CHAMBER.

A.P. Bode, M.S. Read, R.L. Reddick; Dept. of Pathology, East Carolina Univ., Greenville, NC, and The Univ. of North Carolina at Chapel Hill, NC.

We have previously reported on the physical integrity and in vitro responsiveness of human platelets (plts) after stabilization and lyophilization (AABB Abstracts, p.137, 1990). In current studies, a Baumgartner-type whole blood perfusion chamber was used to evaluate the ability of rehydrated freeze-dried plts to adhere to deendothelialized vessel strips and/or become activated. The perfusion chamber was prepared by everting two 1 cm strips of rinsed, denuded canine arterial blood vessel on a tapered rod. Rehydrated plts were combined with fresh packed RBC and plt-free plasma and compared against fresh WB. Adherence was assessed by epi-fluorescence of plts on the vessel strips as detected with FITC-labelled P2 MoAb to GPIIbIIIa. Evidence of activation of plts during recirculation was evaluated by flow cytometry of non-adherent cells with FITC-labelled CD62 or GP53 MoAbs.

In eight runs, fresh plts covered 53-76% of the vessel surface while rehydrated plt preps ranged from 23-80% coverage. The number of rehydrated plts positive for CD62 or GP53 increased two-fold or more during each perfusion study. Preps with no hypotonic shock response showed no activation. We conclude that rehydrated plts are adherent and activatable much like fresh plts.

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TRANSFUSED REHYDRATED PLATELETS SUPPORT HEMOSTASIS AND THROMBOSIS. M.S. Read, R.L. Reddick,* T.C. Nichols,* D.A. Bellinger,* A.P. Rode, K.K. Taylor,* K.M. Brinkhous, and T.R. Griggs.* University of North Carolina, Department of Pathology and Department of Medicine, Chapel Hill, NC., East Carolina University, Department of Pathology, Greenville, NC.

Fixed, dried, rehydrated platelets (RP) were developed and tested in vitro and in animals for hemostatic and thrombotic efficacy. In an annular perfusion chamber at high shear rates, RP adhere in a monolayer and in clumps on thrombogenic surfaces similar to adhesion patterns seen with fresh platelets. Scanning electron microscopy of formvar grids show fully spread RP with some centralization of cytoplasmic granules. RP surface GPIb and GPIIb/IIIa as measured by flow cytometry with monoclonal antibodies SZ-2 and AN51 specific for human GP1b and 10E5 specific for GPIIb/IIIa showed > 82 % platelets reactive for GPIb and 98 % reactive for GPIIb/IIIa. Rehydrated platelets were labeled with fluorescence dye (PKH26-GL, Sigma) and infused into three normal and one von Willebrand factor deficient (vWD) dog (20 - 80 % of animal's total platelet concentration). Arterial stenosis and injury (Folt's model) was used to induce thrombus formation. Plasma and platelets collected every 30 min during the experiment documented the absence of consumptive coagulopathy and the presence of circulating labeled RP. Fluorescence micrographs showed labeled RP present in thrombi within the lumen, adherent to the subendothelium at sites of internal elastic lamina disruption and present in hemorrhage into areas with deep vessel wall injury. There were no platelets adhering to uninjured endothelium. Saline bleeding times remained in the normal range in the normal dogs and were shortened from >15 min to 7 min in a vWD dog given both rehydrated platelets and canine cryoprecipitate to raise the vWF activity level to ≅ 30%. In both normal and vWD dogs, labeled rehydrated platelets adhered to the cut surface of ear bleeding time wounds and were present in clots formed at the wound site. We have demonstrated that RP retain 1) functional platelet membrane glycoproteins, 2) the capacity to adhere and spread on foreign surfaces, 3) the capacity to adhere to exposed subendothelium at high shear, and 4) the ability to support hemostasis and thrombosis in animal models.

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HEMOSTATIC PROPERTIES OF LYOPHILIZED PLATELETS IN TESTS OF BLEEDING TIMES.

Arthur P. Bode, Marjorie S. Read, Robert M. Lust. Depts. of Pathology and Surgery, East Carolina Univ., Greenville, NC, and Dept. Pathology, Univ. of N.C. at Chapel Hill.

Background: We have previously shown that lyophilized platelets (L-Plt) retain properties of adhesion and activatability in the Baumgartner perfusion chamber (Trans 33:72S, 1993). Now we have analyzed L-Plt in two systems directly testing hemostatic function. **Study Design:** One is a prototype device simulating the Ivy bleeding time in vitro (IVBT) and collagen-induced thrombus formation (CITF) in recalcified whole blood (Xylum Clot Signature Analyzer: CSA); the other is an in vivo bleeding time in dogs on full clinical heart-lung bypass before and after infusion of L-Plt. **Results:** On the CSA, L-Plt gave an average (n=4) IVBT of 1 min 58 sec and a CITF of 73% versus 2 min 14 sec and 88% respectively for fresh platelets. Expired platelet concentrates gave indeterminate results because aggregates clogged the lines. IVBT > 6 min and CITF < 25% is typical of vWD patients. In two canine heart-lung bypass studies, the in vivo bleeding time improved from >15 min to 5-7 min after infusion of a bolus of 2-3 x10^{11} L-Plt. The corrected count increments were (#1) 88% and (#2) 47% based on estimated circulatory volume. **Conclusions:** These results demonstrate the hemostatic activity of L-Plt and their potential value in transfusion medicine.

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HEMOSTATIC PROPERTIES OF HUMAN LYOPHILIZED PLATELETS IN A THROMBOCYTOPENIC RABBIT MODEL AND A SIMULATED BLEEDING TIME DEVICE.

A. P. Bode, M. Blajchman, L. Bardossy*, and M.S. Read, Departments of Pathology, East Carolina University, Greenville, NC, and McMaster University, Hamilton, Ontario, and University of North Carolina at Chapel Hill, NC.

We have shown previously that our preparations of lyophilized human platelets (Lyo-Plt) are structurally intact upon reconstitution, and that they adhere to thrombogenic surfaces in vitro and in vivo (Blood 82:159a, 1993). Now we have tested the ability of these preparations to correct the ear bleeding time (BT) in rabbits made thrombocytopenic and immunosuppressed as detailed elsewhere (Blood 82:3489, 1993). Also, we have measured the in vitro bleeding time (IVBT) and collagen-induced thrombus formation (CITF) of Lyo-Plt in a prototype in vitro bleeding time device called the Clot Signature Analyzer (CSA, Xylum Corp, NY). In the rabbits, the endogenous platelet count was $\leq 10 \times 10^6$/mL and the BT was ≥ 900 seconds before infusion of 40-50 x 10^9 platelets. A platelet count and duplicate BTs were then performed one hour after infusion. For the CSA test, platelets were resuspended in fresh citrated plasma at 300-500 x 10^6/mL and combined with an equal volume of washed RBC to remake whole blood. $CaCl_2$ was added to 5 mM in the blood just prior to initiating each run. Mean results for Lyo-Plt versus human platelet-rich plasma (fresh) or 5-8 day old expired blood bank platelet concentrates (Exp PC) are tabulated below:

	Rabbit Model				Clot Signature Analyzer		
	n	% Recovery	Count	Ear BT	n	IVBT	CITF
Lyo-Plt	(12)	58%	108	234 sec.	(4)	118 sec.	73%
Fresh	(6)	79%	153	177 sec.	(4)	134 sec.	88%
Exp PC	(0)	ND	ND	ND	(3)	281 sec.	39%

The Lyo-Plt had reduced 1 hour recovery in the rabbit model relative to fresh platelets, but the mean BT result was similar to the value seen in non-infused rabbit controls with equivalent endogenous platelet counts. In the CSA, the Lyo-Plt gave results similar to that of fresh platelets and better than that of stored platelets in the IVBT and the CITF (t-test, $p < 0.05$). These findings support the notion that Lyo-Plts are hemostatically active.

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PROMOTION OF PLATELET ADHESION TO THROMBOGENIC SITES BY MICROPARTICLES IN STORED PLATELET CONCENTRATES.

L-Y. Yang,* and A.P. Bode, Department of Pathology, East Carolina University, Greenville, NC.

During storage of platelet concentrates (PC) in the blood bank, platelet-derived microparticles (PDMP) appear in the supernatant plasma. The hemostatic value of PDMP in transfusions has yet to be determined, in part because of the difficulty in defining and purifying PDMP. We have carried out a qualitative test of PDMP function in the Baumgartner perfusion chamber (Baum) and in a prototype bleeding time device called the Clot Signature Analyzer (CSA) (Xylum Corp., NY) by comparing results with PDMP-rich supernatant plasma from outdated PC before and after removal of PDMP by filtration of the plasma through a 0.2 micron Acrodisc filter. This filtration lowered PDMP counts as assessed by flow cytometry by >90%. The Baum experiments were performed with fresh platelets or platelets from expired PC resuspended in filtered or non-filtered plasma from expired PC, mixed with an equal volume of washed RBC. The CSA experiments were performed on the same recombined whole blood samples immediately after recalcification with 5 mM $CaCl_2$. The results from the Baum runs were quantified as the percent of coverage of surface area of the denuded vessel strip in the chamber as revealed by immunofluorescence of adherent platelets under epifluorescent microscopy. The CSA measures platelet adhesion and hemostatic plug formation in vitro with several parameters, designated H1, H2, and IVBT (in vitro bleeding time); platelet procoagulant activity and collagen interaction are related to the parameters designated CT1, CT2, and CITF (collagen-induced thrombus formation). Mean results (n=9) for selected parameters are presented below:

	BAUM % Coverage	CSA IVBT	CSA CITF	PDMP x10^6/mL
Non-Filtered	60%	99 sec.	68%	62
Filtered	34%	215 sec.	51%	5
paired t-test	p=0.01	p=0.07	p=0.04	p=0.004

For all parameters, platelets in PDMP-poor filtered plasma gave values signifying poorer adhesion or function versus the same platelets in PDMP-rich non-filtered plasma. H2 and CT1 did not achieve statistical significance in the paired t-test because of a high degree of variance in the determinations. However, in the Sign test all differences showed statistical significance at $p \leq 0.05$. We believe these findings demonstrate a positive effect of PDMP on adhesion of platelets and their hemostatic function as assessed in these tests.

Lyophilized platelet response with microparticles also added to Poster at time of presentation

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A STABLE RADIOLABEL FOR FRESH AND DRIED PLATELETS. RJ Kowalsky, KK Taylor, DK McMahon, ME Brecher, DA Bellinger, RL Reddick, MS Read. University of North Carolina, Departments of Pathology, Pharmacy, and Radiology, Chapel Hill, NC.

In previous studies dried rehydrated platelets retained hemostatic properties and behaved like fresh platelets in animal models (PNAS submitted). However, dried platelets, unlike fresh platelets, did not retain a radiolabel. A stable radiolabel has been developed for fresh and stabilized lyophilized rehydrated canine platelets. ^{111}In-Tropolone, ^{51}Cr-Sodium Chromate, ^{125}I-NaI with Iodogen or Iodobeads, and ^{125}I-PKH95 were examined regarding labeling efficiency (LE), label stability over time in normal dog plasma or buffer, and botrocetin stimulation following radiolabeling. The table below indicates that ^{111}In-Tropolone and ^{125}I-PKH95 produced the highest LE. The high LE of ^{111}In-Tropolone, however, is compromised by poor label stability during incubation in NDP, with label translocation from platelets to plasma protein. The low LE and instability of ^{51}Cr and ^{125}I-NaI labels make them unsuitable platelet labels. Only ^{125}I-PKH95 provides satisfactory LE and label stability. Higher LE is achieved when mfr supplied buffer is used during labeling, however, this buffer causes aggregation of fresh platelets. Mfr buffer did not affect the LE, stability or botrocetin-induced aggregation of dried platelets. Substitution of PBS as the labeling buffer with ^{125}I-PKH95 lowered the LE but did not promote aggregation of fresh platelets during the labeling reaction. Both fresh and dried canine platelets labeled with ^{125}I-PKH95 in PBS demonstrate stable radiolabeled platelets that undergo botrocetin stimulated aggregation following labeling and warrants further consideration as a label for in vivo platelet survival studies.

Radiolabel	Percent LE Fresh	Percent LE Dried	Percent Bound Over Time * Fresh	Percent Bound Over Time * Dried
^{111}In-Trop	94	88	98 (16 hr) B	98 (16 hr) B
^{111}In-Trop	97	88	73 (20 hr) N	26 (6 hr) N
^{51}Cr-Sod Chr	19-22	0	28 (21 hr) N	--
^{125}I-Iodogen	2-9	19	46 (18 hr) N	48 (15 hr) B
^{125}I-Iodobeads	7-23	3	30 (18 hr) N	33 (5 hr) N
^{125}I-PKH95 (Mfr Buffer)	--	89-94	--	91 (22 hr) N 89 (72 hr) N
^{125}I-PKH95 (PBS)	67	65	93 (23 hr) N	97 (24 hr) N

* N = normal dog plasma @ 37°C B = ACD/Saline Buffer @ Rm Temp

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LONG-TERM STABILITY OF LYOPHILIZED PLATELETS: PHYSICAL INTEGRITY AND HEMOSTATIC FUNCTION..

Arthur P. Bode and Marjorie S. Read Depts. of Pathology, East Carolina Univ., Greenville, NC, and Univ. of N.C. at Chapel Hill.

Background: We have presented data (PNAS 92:397-401, 1995) that human and animal platelets lightly treated with paraformaldehyde remain intact and function hemostatically after lyophilization and rehydration. The stability of these platelets in the dried state is now under study. **Study Design:**Aliquots of four dried preparations (2 mL each @ 1-2 x 10^9/mL) were placed in desiccators at 22°C or 4°C or at -70°C and rehydrated with Unisol for testing at 6 and 12 months. Tests included integrity of surface glycoproteins by flow cytometry, ristocetin-induced agglutination, morphology score, and in vitro hemostatic function assessed with the XYLUM Clot Signature Analyzer (CSA). **Results:**Dried platelets stored at 22°C showed 20-40% loss of GPIb and GPIX, nearly complete loss of ristocetin response, and deterioration of morphology score. Platelets stored at 4°C or at -70°C were essentially unchanged. The CSA showed that the 4°C and -70°C platelets gave normal in vitro bleeding times(0:24 -3:22) and normal collagen interactions times (8:30-13:30). **Conclusions:** These results demonstrate retention of important hemostatic function characteristics of platelets stored in the dried state for up to one year.

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Dehydration of Platelets and Red Blood Cells: Long Term Storage of Transfusion Products.
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A. The primary objective of the above study was to explore the role of rehydrated platelets in hemostasis and thrombosis. There were four specific aims:

1. To test the ability of rehydrated platelets to support hemostasis by supporting the production of thrombin and formation of a platelet plug.

2. To study the effects of transfusions of rehydrated platelets on plasma coagulation factor activity as a measure of prethrombotic activity. These studies were designed to look for evidence of abnormal thrombosis and disseminated intravascular coagulation.

3. To study multitransfused animals for evidence of antiplatelet antibody production.

4. To examine the effects of storage on rehydrated platelets.

The highlights of the scientific accomplishments are:

1. We have developed a procedure for lyophilizing platelets that retain many hemostatic properties after rehydration.

2. We have developed an assay for testing production of thrombin based on presentation of surface procoagulant lipids. Rehydrated lyophilized platelets (RL platlelets) appear more activated than fresh platelets and this partial activation may be the mechanism contributing to the hemostatic action. Infusions of these lyophilized platelets caused no change in coagulation factors VIII and IX, no change in circulating fibrinogen and there was no evidence of fibrinogen degradation products.

3. Although RL platelets alone will not aggregate in response to ADP or thrombin in an aggregometer, they are incorporated into aggregates of fresh platelets induced by ADP, thrombin and collagen suggesting that transfused RL platelets may play a role in controlling bleeding in thrombocytopenic patients and in patients with platelet

deficiencies.

4. We have developed animal models of thrombocytopenia for testing rehydrated lyophilized platelets. The infusion of rat antithrombocyte antibodies causes a reduction or depletion of rat platelets that is dose dependent. We have used this model to demonstrate that infusions of rehydrated platelets will correct a long bleeding time in thrombocytopenic rats.

5. Antibodies raised in rabbits against RL platelets do not distinguish between fresh and RL platelets. This suggests that the surface proteins of RL platelets are not remarkably different from those of fresh platelets. Neo-antigens appearing on the surface of RL platelets would be expected to generate antibodies for specific recognition of RL platelets. This is not the case.

6. Storage of RL platelets for one year does not increase procoagulant activity of RL platelets.

7. RL platelets are hemostatic and participate in platelet plug formation. When vessels are injured, rehydrated platelets adhere to the subendothelium and form clumps of platelets. Rehydrated platelets are incorporated in small microthrombi formed in the lumen of deeply injured vessels.

These data strongly suggest that RL platelets may be a valuable tool to stop abnormal bleeding. Further study is needed to fully characterize the mechanisms whereby RL platelets support hemostasis. Additional studies must be done to establish half-life and recovery in animal models.

<u>Studies conducted during the last year</u>.

1. Translocation of procoagulant lipid phosphatidyl serine (PS) to the outer membrane of RL platelets.

2. Use of thrombocytopenic rats to evaluate hemostatic properties of various preparations of RL platelets expressing PS on the membrane.

Studies conducted during the course of this grant.

Hemostatic and thrombotic potential of rehydrated platelets.

Platelets provide the procoagulant surface for the assembly of the tenase-prothrombinase complex. In order for rehydrated platelets to be effective hemostatic agents, they must support thrombin production. Procoagulant phosphatidyl serine on the inner leaflet of the platelet must be transported to the outer membrane surface. We have modified assays developed by Wagenvoord at al (Thrombosis and Hemostasis, 72:582,1994) to measure thrombin production dependent on the presence of activated RL platelets. The presence of PS on the surface of the platelets limits the prothrombinase activity. Equal concentrations of dried and fresh platelets were treated with thrombin, collagen, or thrombin and collagen. A chromogenic assay was developed to measure conversion of added prothrombin to thrombin which indirectly tests for surface bound PS. Rehydrated platelets were found to be more activated than fresh platelet as judged by their ability to convert added prothrombin to thrombin. Fresh washed platelets did not convert prothrombin to thrombin unless they were first stimulated by exposure to collagen or non-aggregating levels of thrombin. RL platelets, on the other hand, were capable of prothrombin conversion without addition of activating agents (Fig.1). However, rehydrated platelets were capable of further activation by incubation of RL platelets with the activating agents. This is an exciting finding, in that it suggests that fixed, dried, rehydrated platelets may retain some metabolic activity. This partial activation may be one mechanism that mediates normal hemostasis in thrombocytopenic animals infused with rehydrated platelets. If procoagulant lipids are rate limiting for the prothrombinase activity then our ability to control the so-called flip-flop reaction may enable us to produce platelets with better circulation time. Expression of procoagulant phospholipids should allow us to compare the various preparations of platelets and provide a quality control test for platelet function and thrombosis risks.

Although our data to date indicate that RL platelets as presently prepared are somewhat activated and capable of conversion of prothrombin to thrombin, in animal models, we have no evidence of abnormal thrombosis or abnormal thrombin generation. Tests were conducted for conversion of coagulation

factors VIII, IX, and X to the activated form along with a bank of tests including activated partial thromboplastin time, prothrombin time and clotting time. We found no evidence of coagulation factor activation and no evidence of disseminated intravascular coagulation after treatment in vivo with RL platelets.

In vitro, the only variable was the shortening of the plasma clotting time with RL platelets substituted for fresh platelets. However, in tests of the plasma incubated with RL platelets without added calcium, there was no evidence of factor activation, and no evidence of fibrinogen conversion to fibrin. Even though the RL platelets have PS on the surface, indicating partial activation, there is no conversion of prothrombin to thrombin without added FXa-FVa-Ca to start the prothrombinase.

Rat model of thrombocytopenia.

A rat model of thrombocytopenia has been developed (see enclosed PNAS publication). The use of an antibody to remove or reduce platelets proved more humane and efficient than induction of thrombocytopenia by whole body irradiation or chemical induction. In our rat model, most of the RL platelets lots were hemostatic. Failures of RL platelets to correct bleeding in the thrombocytopenic rat were usually due to our failure to raise the platelet level to that required with fresh platelets for normal hemostasis. The over all results were very favorable. These studies point to our need for more in depth studies on circulating ability of RL platelets.

RL platelet aggregation.

When RL platelets were tested in the aggregometer with mixtures of plasma and ADP or plasma and collagen, no large aggregates formed. There were some indications that small aggregates formed, but that was not a consistent finding. Platelet aggregation by ADP is induced through the glycoprotein IIb/IIIa receptor (GPIIb/IIIa). This receptor has been shown to be present on the surface of RL platelets. GP IIb/IIIa plays a key role in binding fibrinogen thus linking platelet to platelet and securing the platelet-fibrin plug. We have morphologic evidence of fibrin attachment to the RL platelet surface, but no highly reproducible evidence of radiolabeled fibrinogen binding. These labeled fibrinogen studies are under way in our laboratory. The variability of binding may be experimental error or variability in platelet product. This is a crucial issue and requires continued study.

When mixtures of fresh platelets and RL platelets were stimulated with ADP or collagen, large aggregates formed which contained as many or more RL platelets as fresh platelets. This is a significant finding since few if any patients have no platelets and the few remaining platelets will recruit RL platelets in hemostatic events.

Animal model of hemostasis and thrombosis.

During the course of this grant, studies were conducted in animals to confirm that rehydrated platelets adhere only to injured surfaces and not to normal vessel wall endothelium. These studies were performed in both pigs and dogs, normal and bleeder animals. Adhesion of both normal and rehydrated platelets was minimal when von Willebrand factor protein was absent, a finding reported in the original study for the development of lyophilized platelets.

Platelet adhesion and spread on vessel surfaces is required for normal hemostasis. We use an ex vivo Baumgartner procedure to study adherence and spread of rehydrated platelets on subendothelial vessel surfaces. Porcine vessels were collected and everted over rods mounted in a closed chamber. These vessels are stripped of endothelial cells and present a thrombogenic subendothelial surface. Anticoagulated whole blood is pumped at a controlled rate over the everted vessels. The vessels are then removed and prepared for transmission electron microscopy (TEM). TEM allows for the viewing and evaluation of the number of platelets adherent to the thrombogenic surface. The number of adherent platelets and the degree of activation as measured by pseudopod formation and spreading on the surface allow us to compare the response of rehydrated platelets and fresh platelets. Samples of whole blood were prepared with RL platelets substituted for fresh platelets. Rehydrated platelets mimic fresh platelets in this test system. Nearly equal numbers of platelets adhere. Both fresh and rehydrated platelets form pseudopods and spread. Rehydrated platelets spread at a slower rate than fresh platelets (see figure 2, page 399, PNAS 92, 1995, enclosed). Rehydrated platelets display less shape change but are in the process of shape change and spreading. Our studies indicate that rehydrated platelets respond like fresh platelets but at a slower rate. Fig. 2, C and D in the above cited article are photomicrographs of spread fixed and fresh platelets.

Transfusions studies.

Studies were reported in detail in the 1993 annual report on dogs transfused with rehydrated platelets. Blood samples were collected and analyzed weekly for three weeks following RL platelet infusions and no platelet antibodies were detected. No animals suffered thrombocytopenia. There was a decrease in platelets in some animal immediately post transfusion which was prevented by use of leukocyte filters. In none of our studies have we seen platelet antibodies produced against canine platelets in the dog, either autologous or heterologous.

Storage effects.

Studies relating to storage are ongoing. We see no evidence of one year storage activation as measured in the above assay for conversion of prothrombin to thrombin.

Publications.

1. Read, M.S., Khandelwal, G., Miller, R.S., Taylor, K.K., Bode, A.P., Nichols, T.C., Bellinger, D.A., Griggs, T.R. ,& Brinkhous, K.M. (1995) Rehydrated lyophilized platelets: a proposed mechanism contributing to the hemostatic action, *Blood* 86, 3616 (abstr.)

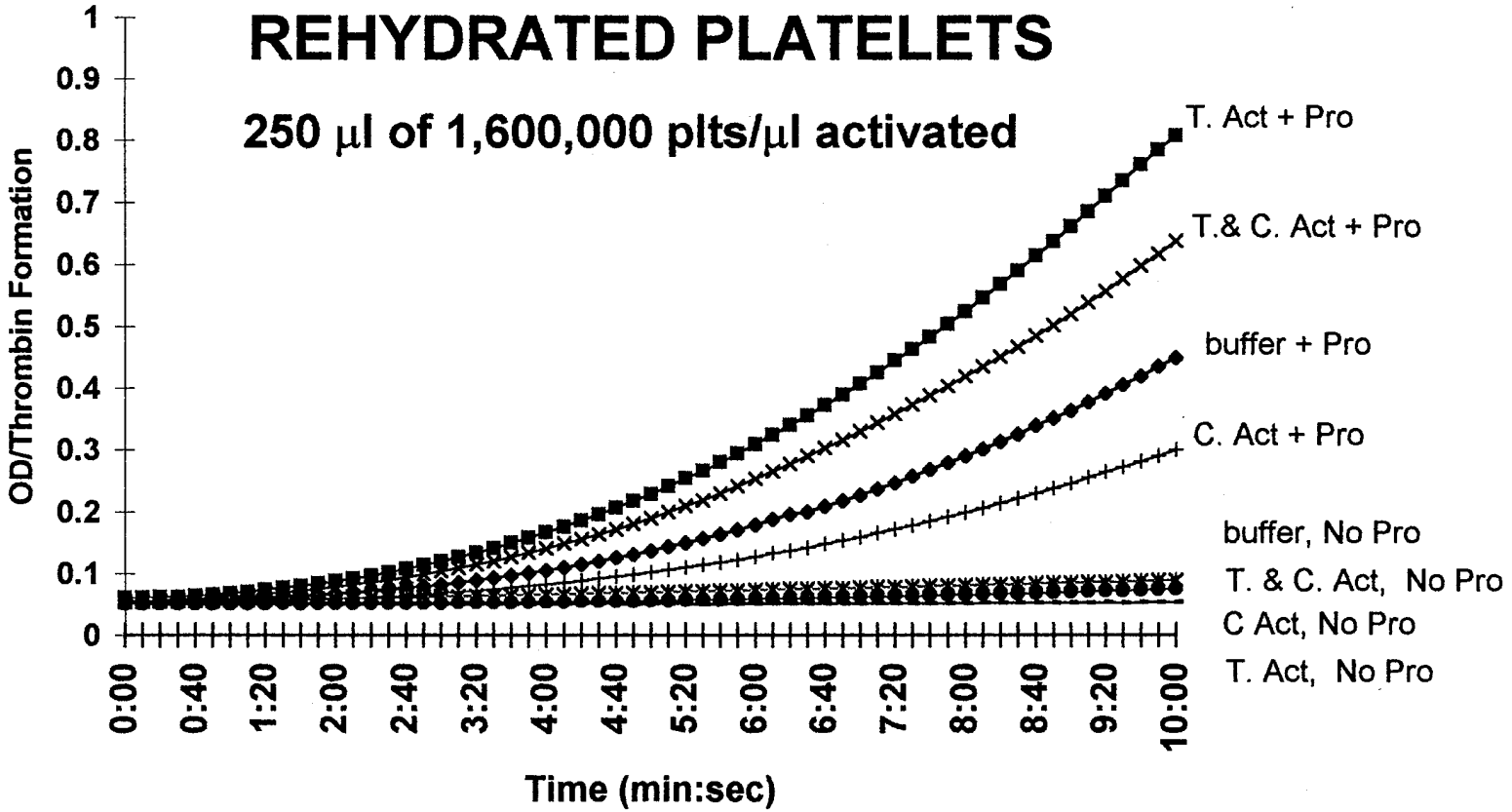
2. Read, M.S. & Bode, A.P. (1995) Platelet storage: efforts to extend the shelf life of platelet concentrates, *Molecular Medicine Today* 1, 322-328.

3. Read, M.S., Reddick, R.L., Bode, A.P., Bellinger, D.A., Nichols, T.C., Taylor, K.K., Smith, S.V., McMahon, D.K., Griggs, T.R., & Brinkhous K.M. (1995) Preservation of hemostatic and structural properties of rehydrated lyophilized platelets: potential for long-term storage of dried platelets for transfusion, *Proc. Natl. Acad. Sci.* 92, 397-401

4. Kowalsky, R.J., Taylor, K.K., McMahon, D.K., Brecher, M.E., Bellinger, D.A., Reddick R.L., & Read, M.S. (1994) A stable radiolabel for fresh and dried platelets, *Blood* 84, 1278 (abstr.)

5. Read, M.S., Redick R.L., Nichols, T.C., Bellinger, D.A., Bode, A.P. Taylor K.K., Brinkhous, K.M. & Griggs, T.R. Transfused rehydrated platelets support hemostasis and thrombosis, (1993) *Blood* 82, 623 (abstr.)

Fig. 1



Rehydrated, washed platelets were incubated in one of the following: (1) Thrombin,T (2) Thrombin & Collagen, T & C (3) Collagen, C (4) buffer. Post incubation the platelets were separated from the agonists by centrifugation and washed 1 time.

The platelet pellet was resuspended in either Prothrombin, P, or buffer, No Pro. The FXa-FVa-Ca++ was added to start the prothrombinase activity. Finally the chromogenic agent, S2238, was added and the optical density, O.D., was determined.

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3614

MORPHOLOGIC RELATIONSHIPS AMONG TYPE VI COLLAGEN AND FIBRILLIN MICROFIBRILS AND VON WILLEBRAND FACTOR IN VASCULAR SUBENDOTHELIUM. Jacob H. Rand, Xiao-Xuan Wu,* Ronald E. Gordon* and Ronald R. Uson,* Depts. of Medicine and Pathology, Mount Sinai School of Medicine, New York, NY.

von Willebrand factor (vWF) is secreted by vascular endothelial cells into the subendothelium (SE) where it plays a role in promoting platelet adhesion. To which SE constituent(s) is vWF bound? We previously showed vWF colocalizes with collagen VI in SE. However, others have suggested that non-collagen VI microfibrils - presumably elastin-associated fibrillin - are the actual binding site. We therefore investigated the relationships among vWF and the two microfibrillar proteins, collagen VI and fibrillin in SE of human umbilical blood vessels. Single and double label immunoelectron microscopy were performed on tissues which were incubated with antibodies using a pre-embedment protocol. Incubations with rabbit polyclonal anti-vWF antibodies, monoclonal anti-collagen VI, and monoclonal antifibrillin were followed by the species appropriate gold-conjugated second antibodies or protein A-gold. Incubations with equivalent concentrations of non-immune primary antibodies served as negative controls. We found that electron dense vWF-labeled aggregates are associated with collagen VI microfibrils but not with fibrillin microfibrils or with fibrillar collagen. Antifibrillin-labeled microfibrils are usually associated with amorphous cores of internal elastic lamina and colocalize with collagen VI, but not with vWF.

These results are consistent with the hypothesis that collagen VI microfibrils serve as a physiologically important binding site for vWF in SE, where complexes of these two proteins may modulate the hemostatic response following vascular injury.

3615

STEM CELL TYROSINE KINASE-1 LIGAND (STK-1L) DOES NOT STIMULATE HUMAN MEGAKARYOCYTOPOIESIS IN VITRO. M.Z. Ratajczak, J. Ratajczak*, W. Marlicz*, J. Ford*, R. Kregenow* and A.M. Gewirtz. Departments of Pathology and Internal Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA.

Receptors with intrinsic tyrosine kinase activity, and their respective ligands, play an important role in hematopoietic cell development. A newer receptor/ligand member of this family, STK-1 (or Flk2/Flt3)/STK-1L, has been shown to influence hematopoietic cell development, in particular at the early stem/progenitor cell level. Alone, STK-1L appears to have little or no effect on in vitro progenitor cell growth itself but when employed with KL, IL-3, GM-CSF, or EPO, it co-stimulates proliferation of myeloid and B cell, but not erythroid, progenitors. The effect of STK-1L on human megakaryocyte (MEG) development in vitro has not been reported. To address this question, we exposed normal human CD34+ marrow cells, derived from 3 separate donors, to recombinant human STK-1L alone, or combined with other cytokines. Cells were plated in plasma clots and resulting MEG colonies were scored for number, size, and maturation after immunofluorescent identification with a gpIIb/IIIa MoAb. STK-1L (25 ng/ml or 100 ng/ml) alone failed to support either CFU-MEG or BFU-MEG colony formation. When used as a co-stimulator with TPO (50 ng/ml), or [EPO (5U/ml)+IL-3 (20 U/ml)+IL-6 (40 U/ml)], it failed to augment colony formation over that obtained with the various cytokine controls.

STK-1L CONC	EPO+ IL-3+IL-6	TPO
0 ng/ml	77 ± 26*	74 ± 25
25 ng/ml	66 ± 31	65 ± 28
100 ng/ml	66 ± 34	61 ± 23

*CFU-MEG Colonies/10^4 CD34+ cells [mean±SD of 4-6 cultures]

STK-1L's utility for in vitro expansion of CFU-MEG from CD34+ cells in liquid culture was also investigated. As employed, STK-1L failed to influence CFU-MEG production. To explore the possibility that subtle effects on CFU-MEG may not have been detected in the assays employed, we perturbed STK-1L mRNA expression with oligodeoxynucleotides. Though sequence specific downregulation of STK-1L mRNA was documented, no effect on CFU-MEG development was observed. Finally, neither STK-1, nor STK-1L, mRNA was RT-PCR detectable in normal, freshly isolated mature marrow MEGS. These results suggest that neither STK-1, nor STK-1L, plays a significant physiologic role in regulating human MEG development.

3616

REHYDRATED LYOPHILIZED PLATELETS: A PROPOSED MECHANISM CONTRIBUTING TO THE HEMOSTATIC ACTION M.S. Read, G. Khandelwal,* R.S. Miller,* K.K. Taylor,* A.P. Bode, T.C. Nichols,* R.L. Reddick,* and T.R. Griggs*, Depts. of Path., Univ. of NC, Chapel Hill and East Carolina Univ., Greenville, NC

Rehydrated lyophilized platelets (RL) are structurally intact and retain many features of fresh platelets (FP) (PNAS 92:397, 1995). The mean clotting time (CT) (4 min) of R-PRP (R-PRP was prepared by reconstitution of RL platelets in platelet-free plasma) was shortened as compared to mean CT (6 min) of fresh platelet-rich plasma (PRP), suggesting that RL platelets are more activated than FP. We used a chromogenic test (Thromb. Haemost. 72:582, 1994) to measure conversion of prothrombin to thrombin in mixtures containing either FP or RL platelets. The test mix consists of: platelets with prothrombin, (Pro) or Pro and collagen, (Coll) or Pro and thrombin (Thr), or Pro, Coll and Thr, Table, column 1. Factor Xa-factor Va-Ca^{+2} mixture was added to start the prothrombinase activity. Platelets were removed after each step in their preparation for lyophilization and tested in the above mixture.

Thrombin Generation Test
(OD at 4 min)

Test Mixture	FP	Washed FP	Predried	RL Platelets
Pro	0.015	0.006	0.180	0.149
Pro+Coll	0.198	0.094	0.165	0.133
Pro+Thr	0.258	0.228	0.443	0.497
Pro+Coll+Thr	0.353	0.189	0.341	0.335

RL platelets support prothrombin conversion to thrombin similar to collagen stimulated FP. RL platelets can be further stimulated by thrombin as evidenced in this thrombin generation assay. These studies suggest that RL platelets present a phospholipid surface that will support thrombin generation. This mechanism may play a role in the normalization of hemostasis by RL platelets in thrombocytopenic rats with a long bleeding time.

3617

HUMAN PLATELETS POSSESS THE MOLECULAR MOTORS REQUIRED FOR ACTIVE ORGANELLE MOVEMENT. S.W. Rothwell* and V. Calvert* (spon. by C. Krishnamurti). Walter Reed Army Institute of Research, Washington, D.C.

Human platelets actively endocytose plasma proteins that are subsequently secreted during the activation phase of the coagulation cascade. In other cell types the process of endocytosis and secretion is believed to rely on microtubule-associated ATPases, such as kinesin and dynein, to mediate organelle movement. In our studies of human platelets we have found that platelets contain high concentrations of both kinesin and dynein. Dynein is present at levels (0.5 µg/g tissue) that are nearly comparable to those reported for neuronal tissue. Specific activity of dynein assayed in the presence of microtubules is ≈200 nanomoles/mg/min, a 5-fold activation over the level measured in the absence of microtubules. Immunofluorescence microscopy of resting platelets shows that, while the platelet microtubules are arranged in the coiled hoops typical of the marginal band in the cortical region of the platelet, dynein is distributed throughout the cytoplasm of the platelets. Activation of platelets results in a redistribution of dynein so that the immunofluorescence image now shows an association of this protein with vesicular elements in the cortex of the cells. Fractionation of unactivated platelets shows that kinesin is associated with the particulate fraction but dynein remains in the soluble fraction in platelets. Stimulation of platelets with either thrombin or a combination of ADP and epinepherine causes a partial translocation of dynein from the soluble fraction to the particulate fraction. We have also investigated several post-translational modifications to dynein and tubulin. Dynein heavy chain and several other polypeptides of the dynein complex are phosphorylated upon stimulation of human platelets with thrombin. Immunoblots of platelet tubulin also demonstrate that tyrosinated, acetylated and glutamylated tubulin isoforms are all present in platelets.

Platelet storage: efforts to extend the shelf life of platelet concentrates

Marjorie S. Read and Arthur P. Bode

In transfusion medicine, platelets cannot be replaced by blood substitutes. Circulating platelets must respond quickly to changes in normal blood flow and blood-vessel injury to promote normal hemostasis. Adhesion of platelets at the site of vessel endothelial rupture is mediated through platelet membrane glycoprotein receptors. The integrity of these surface adhesion receptors and the signal-transduction pathways of activation will determine, in large part, how well a platelet functions in hemostasis. The deterioration of these systems during storage leads to a compromise of function known as the 'platelet-storage lesion'.

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THE usefulness of platelet concentrates in transfusion medicine is greatly limited by a short (five day) shelf life. The typical unit in a blood bank is already three or more days old by the time it has passed viral screening and is released for inventory. In a military situation, platelet concentrates are hardly ever made available at aid stations or field hospitals because of the logistical barriers of collecting and transporting platelets to the site of use before their shelf life has expired. Civilian blood banks have frequent periods of shortages of platelet concentrates, when supply cannot keep up with demand and, at other times, large numbers of platelet concentrates are wasted because they cannot be used before their expiration date.

The situation can be improved slightly if specifically cross-matched donors are used for certain patients who require chronic transfusions, thereby reducing the demand on the general stock of platelets. Multiple transfusions may be required for patients with acute leukemia or aplastic anemia, patients receiving bone-marrow transfusions, and other patients receiving chemotherapy. Large volumes of platelet concentrates are prepared by automated apheresis from the directed donor for a specific patient. However, at present, only a small fraction of platelet transfusions are managed in this way.

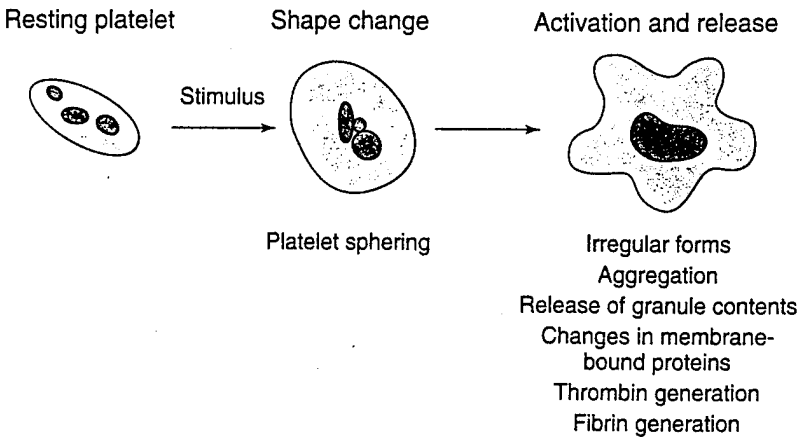


Figure 1. Schematic representation of changes in platelet shape and activation. Exposure of platelets to activating agents such as collagen, thrombin and foreign surfaces results in loss of discoid shape, centralization of granules, aggregation, and secretion and release of granule contents, which further amplifies aggregation.

Platelet-storage lesion

The platelet-storage lesion manifests both as a loss of function and by changes in morphology. Changes in platelet morphology, including loss of discoid shape and degranulation or vacuolization, seen with platelet activation (Fig. 1), can occur during storage and have been associated with decreases in post-transfusional recovery and circulation of stored platelets[1]. Several studies show that morphology is the best predictor of the ability of stored platelets to remain in circulation[1–3]. This correlation has been shown, but no causal relationship has been clearly established.

Platelets in the resting state do not have a high rate of metabolism (Fig. 2). However, with storage, platelets show an increased rate of respiration with accelerated production of lactic acid and a depletion of the ATP pool, consistent with activated platelets[4,5]. Some activation probably does occur during collection and isolation of platelets in plasma. In relation to the storage lesion, depletion of the platelet energy stores may explain the refractory nature of stored platelets to further stimulation. It is possible, however, that the function of stored platelets may be recovered following transfusion as a result of restoration of normal energy stores[6].

Platelet surface glycoprotein Ib (GPIb) is a major platelet receptor, which contributes to *in vivo* platelet adhesion to subendothelial-bound von Willebrand Factor (vWF). It has also been shown to play

Glossary

Alloimmunization – Induction of antibodies that produce a refractory state in patients receiving multiple platelet transfusions. Most of the antibodies are against HLA antigens carried on white blood cells as a blood-product contaminant.

Automated apheresis – A process involving continuous centrifugation and harvest of platelets from a single donor.

Bleeding time – Test of primary hemostasis involving platelet function. A uniform cut is made in the forearm and the time taken for bleeding to stop is recorded as the bleeding time.

Cross matching – Platelets are tested for immuno-compatibility with the recipient to avoid rejection resulting from differences in platelet-specific antigens.

Febrile transfusion reaction – Many recipients of platelet transfusions experience a transient shaking and chills reaction, which is related to the presence of leukocyte antigens or cytokines in the transfusion product.

Hypoxic – Platelets in an oxygen-deficient medium will undergo the Pasteur effect and release lactic acid.

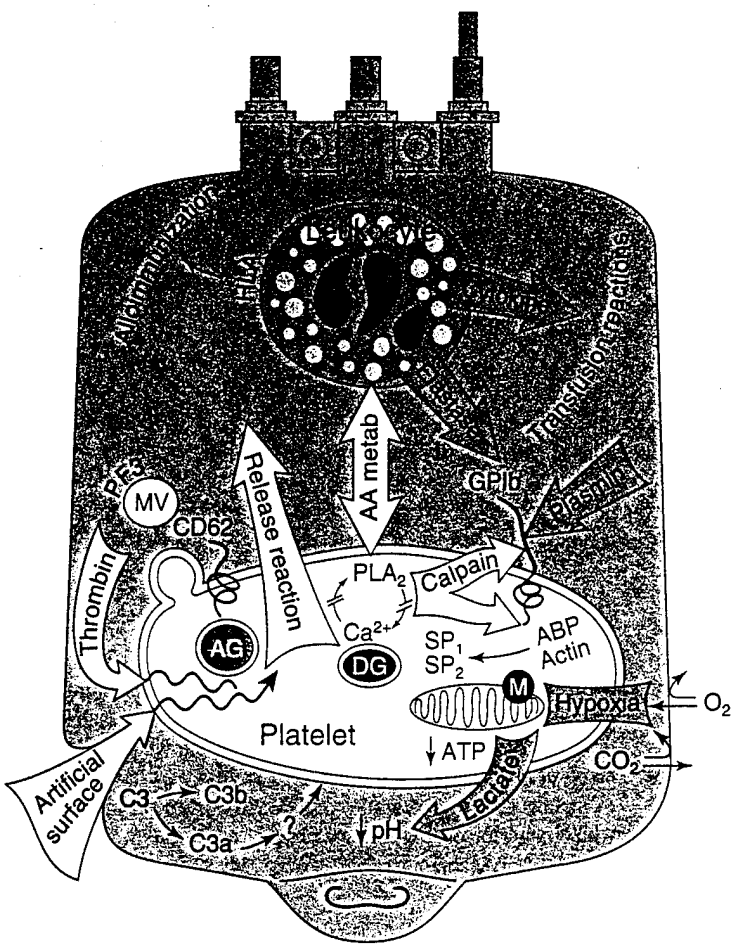
Leuko-reduction – Removal of white blood cells from blood products by filtration through a white-blood-cell-binding resin.

Platelet resting state – Resting platelets are unstimulated cells that have not been activated by agonist or agents that cause calcium flux, release of granule contents or morphological changes.

Refractory state – Failure of transfused platelets to elevate the circulating platelet count. Immediate clearance of transfused platelets is usually due to alloimmunization or, rarely, non-immune causes.

Thrombocytopenia – Markedly reduced or diminished circulating platelets, usually a platelet count of less than 10 000 platelets μl^{-1}.

Unit – One unit of platelet concentrate is 30–50 ml plasma containing approximately $5–10 \times 10^{10}$ platelets.



Facets of storage lesion

- Activation by thrombin, bag surface, arachidonic acid metabolites
- Refractory to further stimulation
- Acceleration of thrombin formation by platelet factor 3 on microvesicles
- Disruption of Ca^{2+} flux and signal transduction
- Degradation of GPIb by calpain, plasmin, elastase
- Hypoxia in older bags, lactic acid generation, metabolic exhaustion, acidification of medium
- Degradation of cytoskeleton by calpain

Related problems

- Alloimmunization through HLA
- Transfusion reactions due to cytokines
- Enzyme activation in plasma, degradation of clotting factors
- Bacterial growth in bag

Figure 2. Major contributing factors in the development of the platelet-storage lesion. *In vitro* mechanisms promoting activation of stored platelets are highlighted in green. The agents or reactions directly damaging platelets are highlighted in pink. The areas not illustrated include the interaction of complement with platelets during storage, and the nature of the refractoriness of stored platelets to further stimulation in Ca^{2+}-dependent pathways. The storage lesion must be regarded as a multifactorial process. Abbreviations: M, mitochondrion; DG, dense granule; AG, alpha granule; MV, microvesicles; AA metab, arachidonic acid metabolites; ABP, actin-binding protein; PF3, platelet factor 3 (procoagulant); PLA_2, phospholipase A_2; GpIb, glycoprotein Ib.

a role in *in vitro* and *ex vivo* vWF-dependent platelet agglutination[7,8]. Adhesion of platelets is essential to prevent bleeding, and is mediated by the interaction of GPIb–V–IX complex[9] on the platelet surface, and von Willebrand Factor (vWF) bound to the subendothelium (Fig. 3). The GPIb–IX receptor-adhesion event may provide an active platelet surface for further hemostatic and thrombotic events.

The absence of platelet GPIb results in a serious bleeding disorder termed the Bernard–Soulier syndrome. While the function of all platelet membrane glycoproteins in hemostasis and thrombosis is complex, GPIb is essential for normal platelet adhesion at high shear in free-flowing blood. In the absence of GPIb, platelets do not adhere properly to damaged vessel walls *in vivo* and bleeding occurs. Surface membrane glycoprotein has been shown to decrease with storage[4,10,11]. Using a quantitative GPIb activity assay, eight out of nine platelet concentrates tested showed less than 15% GPIb activity remaining after storage for 14 days[11]. GPIb lost from the platelet surface, probably by the action of plasmin cleavage, can be replenished from intra-platelet stores. The relocation of GPIb to the surface may be associated with platelet cytoskeleton changes[10]. Preservation of GPIb molecules on stored platelets also has been associated with *in vivo* platelet recovery[12]. Stored platelets appear to be less functional than fresh platelets in *in vitro* tests of ristocetin-induced agglutination in plasma, and by correction of bleeding time *in vivo*. Whether the gradual loss of GPIb during storage correlates with loss of function remains to be demonstrated conclusively.

GPIIb/IIIa expression on the platelet surface is also altered during platelet storage. Loss of GPIIb/IIIa surface expression during storage is rapidly replaced by intracellular stores and, in fact, surface

expression of GPIIb/IIIa actually increases with storage. This increase is probably due to platelet activation and release, with released GPIIb/IIIa relocating to the platelet surface[13,14].

Historical milestones in platelet blood banking

Fresh whole blood cannot be made readily available to a significant degree in modern, high-demand medical centers. Testing for blood type and for bacterial and viral contamination would delay any use of whole blood for at least 24 hours. Storage of whole blood at 4°C was abandoned when blood banks discovered that platelet function rapidly deteriorated in refrigerated whole blood anticoagulated with sodium citrate, perhaps as a consequence of the breakdown of white blood cells during storage. Storage of separate components of blood quickly became the norm.

Storage temperature

Throughout the 1960s and into the early 1970s, platelets were stored in blood banks as a concentrate of platelets in plasma at 4°C. Fresh whole blood collected in citrate anticoagulant was centrifuged to separate platelets and plasma from the red cells, followed by centrifugation of the platelets and resuspension of the platelet pellet in 40–60 ml of supernatant plasma. In 1969, Murphy and Gardner[15] reported studies showing that platelets stored at 22°C remained in the recipient's circulation much longer after infusion than platelets stored at 4°C. This led to numerous studies comparing the function of platelets stored at 22°C versus 4°C (Ref. 16). It was shown that platelets chilled to 4°C rapidly lose their discoid shape, develop a spherical configuration and have reduced post-transfusional survival. Cold storage interferes with microtubule assembly, resulting in loss of discoid shape and the sphering of platelets. Cold storage appeared to prevent α granule release, but survival and recovery were adversely affected[15,17]. The end result was that blood banks began to store platelets at 22°C to take advantage of the improvement in circulatory half-life. It remains debatable whether or not hemostatic function is similarly improved.

Storage container

Storage of platelet concentrates now took on a new scrutiny in the late 1970s and early 1980s. First-generation blood-storage bags of PL-146 or similar plastics were made for storing whole blood or red blood cells. In these standard plastic containers, the plasma pH of platelet concentrates dropped rapidly as the large number of cells in the platelet concentrates produced and secreted lactic acid as a result of hypoxia. The production of lactic acid by platelets exceeded the buffering capacity of the small volume of storage plasma, thus causing a drop in pH. One problem with first-generation storage bags was the inability of the plastic container to allow adequate escape of carbon dioxide (CO_2) from the container and outside oxygen (O_2) to enter the bag. The conversion of

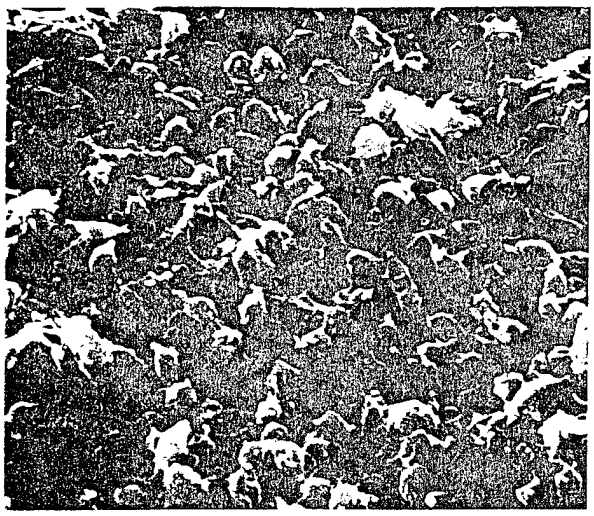


Figure 3. Scanning electron micrograph of platelets adhering to the subendothelium of a denuded vessel wall. In normal vessels, intact endothelium provides a non-thrombogenic surface to flowing blood. Injury to the vascular endothelium exposes flowing blood to subendothelial thrombogenic matrix. Adhesive proteins such as von Willebrand factor and fibrinogen are adsorbed and platelets attach at the injury site.

Figure 4. A typical unit of blood-bank liquid platelet concentrate and one unit of lyophilized platelet concentrate. The platelets were dried in the presence of albumin and appear as a white powder, as displayed.

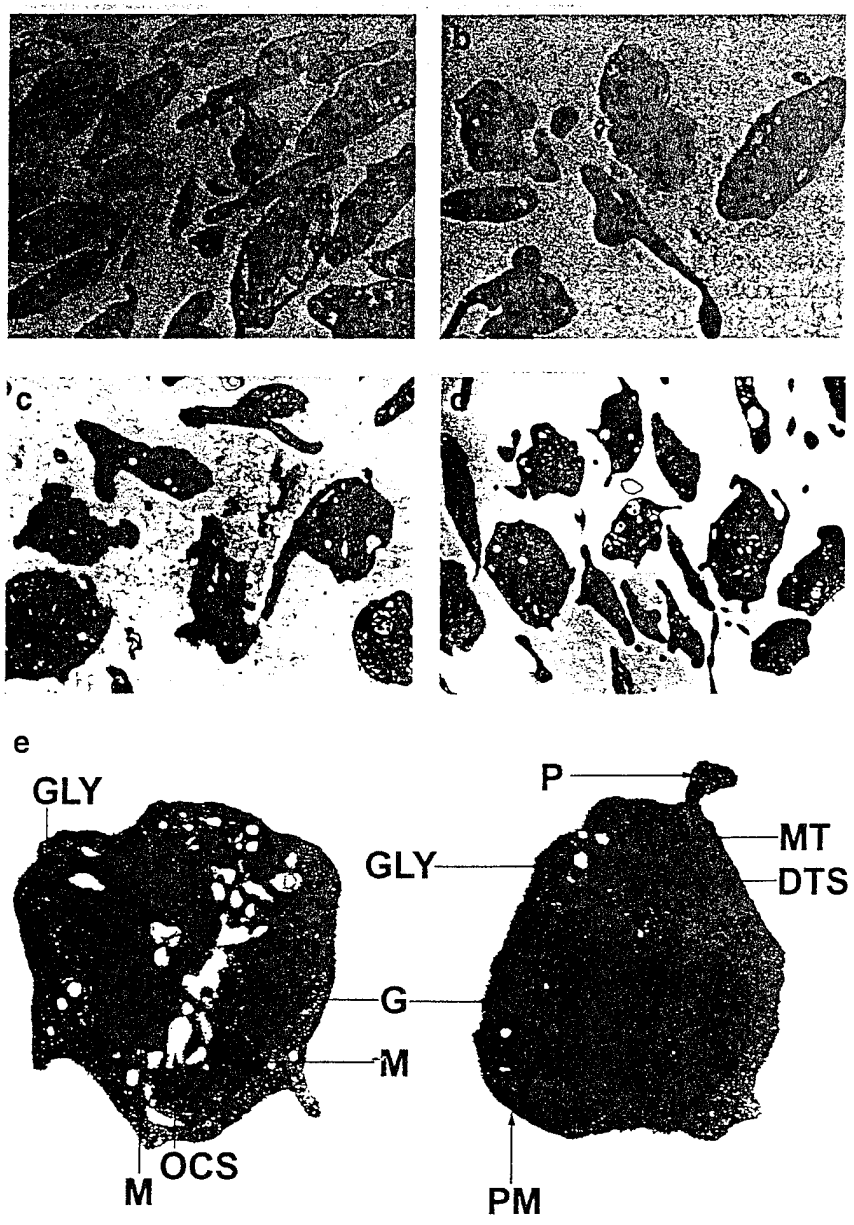


Figure 5. Transmission electron micrographs of platelets. (a) Fresh platelets, stored for one day; (b) platelets stored for five days; (c) platelets stored for seven days; (d) lyophilized, rehydrated platelets; (e) typical ultrastructure of washed fresh platelets. Rehydrated platelets closely resemble washed fresh platelets. Most rehydrated platelets retain a discoid shape with intact membranes, randomly distributed granules and some pseudopod formation.
Abbreviations: GLY, glycogen; M, mitochondrion; OCS, open canalicular system; G, granules; P, pseudopod; MT, microtubules; DTS, dense tubular system; PM, plasma membrane.

glucose to lactic acid and an accumulation of CO_2 in containers with high platelet counts caused a pH drop below 6.5 and platelet swelling occurred[5]. The generation of lactic acid produced by platelet glycolysis was driven by hypoxic conditions in the storage container. Agitation of the bags to prevent platelet activation by cell contact with the surface of the container was of some benefit in reducing hypoxic conditions. However, it was the development of new types of CO_2/O_2 permeable plastics that made it possible to store platelet concentrates for between five and seven days without a fall of pH below 6.2 (Ref. 18).

For a brief period in the early 1980s, the US Food and Drug Administration permitted a seven-day shelf life of platelet concentrates. It soon became apparent that the longer storage time of platelets at 22°C was resulting in an increase in transfusion-transmitted sepsis. The storage period was cut back to five days in 1986 to reduce the danger of bacterial growth. Extensive research into chemical, photochemical, UV irradiation and heat treatment to develop safe and effective antimicrobial treatments of platelet concentrates without unacceptable compromise of function is ongoing[19]. Septic reactions resulting from bacterial contamination are a persistent problem of blood transfusions[20,21].

Storage media

Within this same time frame, and even earlier, the idea of adding platelet-activation inhibitors during the preparation and storage of platelet concentrates in the hope of improving yield, shelf life or viability was tested. Short-acting prostaglandins, such as PGE-1 or PGI-2, which raise intracellular levels of cyclic AMP (cAMP) by stimulating adenylate cyclase, were tested. The total number of platelets collected by centrifugation in concentrates containing inhibitors was much improved[22,23], probably as a result of prevention of platelet activation and clumping during collection and centrifugation. However, the loss of function of platelets during storage was not abated[23]. Forskolin, another compound that elevates cAMP and inhibits platelet activation, was also tested in stored concentrates and found to be only marginally successful[24]. A re-examination of the ability of cAMP to regulate platelet activation showed that phosphodiesterase inhibitors, such as theophylline and caffeine, rather than, or in combination with, adenylate cyclase stimulators were more beneficial and longer lasting in preventing loss of platelet function and integrity during extended storage[25,26]. In autologous infusion studies, platelets stored in a medium containing PGE-1 and theophylline showed improved *in vivo* viability compared with controls, following storage for 14 days[12]. The addition of certain protease inhibitors such as hirudin and aprotinin had an added effect of retaining platelet function during storage, at least by *in vitro* assessment of platelet aggregation, hypotonic shock recovery and platelet morphology[27].

Removal of white blood cells from platelet concentrates appears to have a number of potentially beneficial effects, including, perhaps, a direct improvement in the quality of stored platelets by limiting the exposure of platelets to white blood cell proteolytic enzymes[28]. Leuko-reduction by filtration during blood collection reduces cytokine formation in the concentrates and thus may reduce the incidence or severity of febrile transfusion reactions[29]. The effect of leukodepletion on alloimmunization in chronically transfused patients is less clear and may be related to a threshold concentration of white blood cells[30]. The overall evidence in favor of removing white blood cells from platelet concentrates and from stored red blood cell concentrates has caused white blood cell removal to become a common practice in transfusion centers.

Thus, the deterioration of platelet function during storage appears to have a cause and effect explanation. The acidification and hypoxia were caused by exhaustion of plasma bicarbonate stores, a result of platelet cellular metabolic activity in storage bags containing a large number of cells with inadequate CO_2/O_2 exchange. Improvements have been made by increasing the buffering capacity of plasma, using improved plastics that give the containers better gas permeability, and by adding nutrients such as adenine, dihydroxyacetone and dextrose to the citrate anticoagulant. Effective artificial platelet storage media consisting of buffered and fortified salt solutions rather than plasma have been developed[31–33]. The introduction of platelet-activation inhibitors into the artificial storage medium gave encouraging results, with a possible shelf life for liquid platelet concentrates of two to three weeks[12,25]. These findings suggest that artificial storage media might be superior to plasma for longer term liquid storage of platelet concentrates. There is evidence to support the notion that it is important to inhibit activation of platelets during storage to ensure that the platelet metabolic rate remains low and more easily supported over an extended period of time[8]. The data appear to confirm that the platelet-storage lesion is probably the result of 'metabolic burnout'. The refractoriness is, in part, due to platelet activation during preparation and storage of platelets. Liquid storage of platelets in a truly 'resting state' may allow a shelf life of two to three weeks but, unfortunately, it cannot translate into clinical practice unless combined with an effective antimicrobial strategy.

Modern technology has also provided hope of extending the shelf life of stored platelets. Cryopreservation of platelets at −70°C has been accomplished to give a shelf life of a year or more[34], but with certain penalties, as with red cell cryopreservation. Platelets submerged in 5% dimethyl sulfoxide (DMSO) or glycerol can be slowly cooled to ultra-low temperatures without great loss of cell integrity, but upon thawing, the cells must be washed repeatedly to remove the cryoprotectant agent prior to infusion. There is some evidence that platelet function in those concentrates at the time of infusion is significantly less than that of platelets stored in the conventional manner[35]. Certainly, the expense of storage at −70°C and extra handling costs lessen the appeal of this approach.

Lyophilized platelets

An alternative to cryopreservation and liquid storage of platelets may be lyophilization (Fig. 4). The storage of platelets at room temperature permits bacterial growth[19]. The results of one study[20], reported in 1991, showed that platelet transfusions that induced sepsis increased with increased storage time of multidonor platelet products. There was a fivefold increase in sepsis with platelets stored for five days or longer. The conclusion that transfusion-associated sepsis is problematic and persistent begs for a solution if platelets are ever to be stored for extended periods of time. Freeze-drying of platelets could possibly solve some of the problems associated with preparing a stable product for transfusion medicine, such as bacterial growth in liquid-stored platelets and the requirement for removal of cryopreservatives from frozen concentrates.

The earliest attempts at lyophilization suffered from loss of cell integrity in the processing[36–38]. Recently, platelets treated with paraformaldehyde have been successfully lyophilized and rehydrated to produce a cell preparation that has an intact morphology and preserves many hemostatic properties[39]. A brief incubation of platelets with paraformaldehyde serves to stabilize the platelets and prevent the cellular destruction that occurs with freezing, drying and rehydration in the absence of a cryoprotectant agent. These lyophilized and rehydrated platelets display some alterations in structure, but the changes were not remarkably different from washed fresh platelets, or platelets stored under blood-banking conditions for five days (Fig. 5). Two major glycoprotein (GP) receptors on the rehydrated platelet surface were preserved. By flow cytometry, antibody recognition of GPIb and GPIIb/IIIa was nearly the same as for fresh platelets, with 92% and 98% of lyophilized platelets staining positive for GPIb and GPIIb/IIIa, respectively[39]. These two receptors play a major role in adhesion (GPIb) and platelet aggregation (GPIIb/IIIa).

In an animal model of vascular injury, rehydrated platelets were concentrated at the site of injury and were incorporated into the platelet plug[39]. In an animal model of antibody-induced thrombocytopenia with a prolonged bleeding time, treatment with rehydrated platelets returned the bleeding time to normal[39]. Although these *in vivo* studies were conducted in a small number of animals, the data indicate a potential use of paraformaldehyde treatment as a means for long-term storage of blood platelets. The characterization of rehydrated platelet activity, membrane properties, storage properties, and *in vivo* circulation and recovery remains to be completed.

In vitro (aggregometer) studies showed that rehydrated platelets suspended in plasma do not form aggregates when stimulated with ADP and collagen, but do adhere to collagen fibers. However, in mixtures of fresh and rehydrated platelets in plasma, rehydrated platelets were readily incorporated into the fresh platelet aggregates induced by ADP and collagen. The ability of rehydrated platelets to complement the function of fresh platelets, and participate in

The outstanding questions

- What are the molecular changes in platelets that determine senescence? Is there a specific storage-induced change in GPIb or other surface glycoproteins that triggers early senescence in transfused platelets?
- Which *in vitro* functionality of platelets relates best to competent hemostasis? Do the procoagulant or adhesive properties relate best to *in vivo* function?
- How can all platelet concentrates be made sterile and virus free? Can we eliminate the hazards of transfusion-transmitted disease?
- What will be the evolution of future blood-bank platelet products: extended wet storage, dried storage, or platelet substitutes such as artificial platelets?

aggregation initiated by fresh platelets, may provide a valuable hemostatic aid in patients with low platelet counts.

These studies indicate that lyophilized platelets retain many of the structural and functional properties of stored fresh platelets. Storage in the dry state may alleviate or reduce some of the problems of liquid storage and cryopreservation, such as low yields and high costs. In addition, paraformaldehyde may offer the advantage of being a sterilizing agent, providing a means of storing platelets for several months or even years.

The development of rehydrated platelets is in the initial stages and further investigations are needed to verify the benefits of dried platelets. If this technology can be implemented safely and economically, longer-term storage of platelets in blood banks may become much more convenient in the near future.

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